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The Roots of Plant Frost Hardiness and Tolerance

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Abstract

Frost stress severely affects agriculture and agro-foresty worldwide. While many studies about frost hardening and resistance have been published, most of them focused on the aboveground organs and only a minority specifically targets the roots. However, roots and aboveground tissues have different physiologies and stress response mechanisms. Climate models predict an increase in magnitude and frequency of late frost events which, together with an observed loss of soil insulation, will greatly decrease plant primary production due to damage at the root level. Molecular and metabolic responses inducing root cold hardiness are complex. They involve a variety of processes related to modifications in cell wall composition, maintenance of the cellular homeostasis and the synthesis of primary and secondary metabolites. After a summary of the current climatic models, this review details the specificity of freezing stress at the root level and explores the strategies roots developed to cope with freezing stress. We then describe the level to which roots can be frost hardy, depending on their age, size category and species. After that, we compare the environmental signals inducing cold acclimation and frost hardening in the roots and aboveground organs. Subsequently, we discuss how roots sense cold at a cellular level and briefly describe the following signal transduction pathway, which leads to molecular and metabolic responses associated with frost hardening. Finally, the current options available to increase root frost tolerance are explored and promising lines of future research are discussed.

Keywords

Abiotic stress, Cold acclimation, Frost; Hardiness, Roots, Tolerance

Introduction

Frost is a major meteorological factor impacting agriculture and the agro-forestry economy in temperate climates (Papagiannaki et al., 2014; Snyder and de Melo-Abreu, 2005), as well as in many subtropical regions (Snyder and de Melo-Abreu, 2005), determining the distribution range of many plants and crops. Freezing stress threatens plants integrity by inducing the growth of ice crystals within the plant tissues. However, many plant species from temperate and cold climates can increase their ability to withstand freezing temperatures after being exposed to environmental stimuli such as low temperatures and short days. This complex process, called cold acclimation or frost hardening, is associated with biochemical and physiological changes that ultimately lead to changes in gene expression, osmolyte accumulation and lipid bilayer composition.

Whereas it is widely documented that the climate is changing, frost damage will likely not disappear in a globally warmer climate and may even become more problematic in some regions of the world (Gu et al., 2008). Indeed, while global warming causes milder winter temperatures on average, it also destabilizes polar vortices, thereby increasing temperature variance and the probability of extreme weather events (Francis and Skific, 2015). Furthermore, plants harden less under warmer temperatures, making them less resistant to frost in winter, even if milder (Murray et al., 1994). Taken together, these factors will greatly affect plant distribution and productivity. In most regions, milder winter temperatures will expand the distribution range of species, but also increase the probability of early- and late-frost events (Schwartz et al., 2006). During the last sixty years, global warming hastened bud break and frost dehardening/deacclimation by more than one day per decade (Man et al., 2009; Schwartz et al., 2006). However, plants are the most frost-sensitive just after dehardening and during budbreak, as they as they mobilize reserve resources accumulated during cold acclimation to resume growth (Kalberer et al., 2006). This conjunction of advanced bud break and late frost caused serious damage in Eastern US forests during the springs of 2007 (Augspurger, 2009; Gu et al., 2008) and 2010 (Hufkens et al., 2012). Similarly, in some locations of Southern Europe, climate change and the increased frequency of late frost events shortened the frost-free season by more than 50 days since 1975 (Lavalle et al., 2009). In addition, in order to avoid the deleterious effects of summer drought on grain filling, farmers have to sow crops earlier, increasing the need of frost resistant cultivars and winter varieties (Rezaei et al., 2015). Furthermore, it is expected that global warming will shift species distribution to higher latitudes, sometimes by more than 250 km, where they will be more exposed to extreme weather events (Park et al., 2016).

The precise impact of the increase in CO_2 level accompanying the rise of temperatures is currently a matter of debate. Studies indicate that higher CO_2 concentrations influence the frost resistance in a species-dependent manner. In addition to these changes in maximum frost hardiness, increased CO_2 concentrations have been shown to slow down hardening (Barker et al., 2005) and hasten dehardening (Murray et al., 1994; Repo et al., 1996) of some species, making them more vulnerable to cold spells.

Most studies on frost resistance mechanisms focus on the aboveground organs (Lee et al., 2009; Smékalová et al., 2014). This originates from the difficulty to study roots in their natural environment and the fact that frost damage to the roots appears as a minor concern, as roots are less exposed due to the insulation provided by the soil (Colombo et al., 1995; Regier et al., 2010; Weiser, 1970). However, aboveground and belowground organs have distinct response mechanisms, even at a molecular level (Bigras and Dumais, 2005; Hashimoto and Komatsu, 2007; Ryyppö et al., 2008). As an example, in *Arabidopsis thaliana*, 86% of the cold-induced transcriptome changes are not shared between roots and aboveground organs (Kreps et al., 2002).

Belowground, frost mainly damages and kills fine roots, which are crucial for water and mineral absorption. This costs both time and non-structural carbohydrate reserves to regrow them once conditions improve. This is at the expense of aboveground productivity (Gaul et al., 2008; Man et al., 2009), generally the valued plant parts. Frost also reduces agro-forestry productivity by damaging container-grown seedlings, as their roots are exposed to lower temperatures in containers than they would be in nature (Bigras and Dumais, 2005). Climate change may also increase root frost damage through a reduction of the snow cover and therefore of soil insulation (Comerford et al., 2013) (Figure 1). Gaul et al. (2008) recorded that the temperature of the soil's top 20 cm, where approximately 70% of the fine roots are found, could decrease by up to 5.5°C (from 0.2 to -5.3°C) after manually removing the snow cover in a spruce forest growing on an haplic podzol in South Eastern Germany. Similarly, Cleavitt et al. (2008) reported that in the absence of snow cover, frost propagated 40 cm deeper in a hardwood forest in North Eastern USA (New Hampshire). In addition, roots lacking snow insulation are also more exposed to freeze-thaw cycles. All this can result in significant damage to fine roots, leading to a decrease of the fine root biomass by 50% in boreal forests (Kreyling et al., 2012) and by up to 80% in container grown *Pinus sylvestris* seedlings (Sutinen et al., 1996).

The purpose of this review is to present the current knowledge concerning the impact of frost conditions on roots. The freezing mechanism and the origins of frost damage in plant roots will be discussed. Then, we will

present the environmental signals inducing cold tolerance/frost hardiness of the roots, as well as signal transduction pathway and physiological changes taking place during acclimation. Finally, we will discuss some leads to improve root frost hardiness.

Freezing stress and sources of frost damage

Plants and plant tissues developed two contrasting strategies to cope with freezing stress: tolerate it or avoid it (Burke et al., 1976; Levitt, 1980). Freezing tolerance allows plants and/or their tissues to tolerate the presence of ice in extracellular spaces and the ensuing dehydration stress. On the other hand, freeze avoidance relies on the ability plants to avert the formation of interstitial ice crystals. Although these two strategies are clearly different, they can be found simultaneously in the same plant.

Frost-avoiding tissues can avoid freezing either by insulating themselves from the cold temperatures, or by limiting the formation of ice crystals in their tissues by keeping water liquid below 0°C. The ability to keep water under a liquid state at freezing temperatures is called supercooling (or undercooling) and relies on the fact that the freezing point of pure water, also known as the ice homogeneous nucleation temperature, is at ca. -40°C (Rasmussen and MacKenzie, 1972). However, in nature, water usually freezes at temperatures just below 0°C. This is because in nature, water is nearly always found in association with salts or colloids, under the form of a solution. Some of these salts and colloids have the capacity to act as ice seeds and induce ice-nucleation. These particles are called ice-nucleating agents (INAs) and induce heterogeneous nucleation. Within the root environment, some INAs are synthesized by frost tolerant plants in their extracellular space to allow the initiation of ice nucleation in the apoplast rather than in the symplast. In addition to supercooling, frost-avoiding tissues can further decrease the ice nucleation temperature by synthesizing osmolytes in their fluids, which reduces the water freezing point by 1.86°C per mole of solute (Zachariassen and Kristiansen, 2000). Because of the large amount of solute required to significantly reduce the freezing point, the actual freezing point depression that can be achieved *in planta* is of only 1 to 2°C (Levitt, 1980). Antifreeze proteins, i.e. proteins reducing the freezing point in a non-colligative way, can further reduce the freezing point by up to 0.4°C allowing plants to keep their internal fluids liquid under lower temperatures (Wisniewski and Fuller, 1999).

Due to practical limitations associated with the study of roots in their natural environment, it is currently not known whether roots are frost tolerant or avoidant in their natural environment. However, based on *ex situ*

experiments, it seems that they can be either one, depending on the species studied. The precise ice propagation path in frost-avoiding roots is unknown (Korhonen et al., 2018).

Experiments on the supercooling capacity of aerial organs of monocot plants indicate that their ability to avoid ice formation is poor and it seems to be the same in their roots (Livingston et al., 2016; Stier et al., 2003). For example, in infrared thermography experiments on uprooted herbaceous monocots, ice nucleation was found to always occur in the roots first, followed by the crown and the leaves (Pearce and Fuller, 2001; Stier et al., 2003). Similar observation were made on *in situ* wheat (*Triticum aestivum*) by Livingston et al. (2016). This is expected as soil contains a significant amount of INAs. These INAs can come from plants, fungi, bacteria, decaying matter and various dusts and salts (Hill et al., 2016; Murray et al., 2012). For instance, roots of *Artemisia tridentata* have been found to produce INAs able to initiate ice nucleation at a temperature between -5 and -12°C (Hill et al., 2016). Some soil fungi produce INAs able to initiate ice-nucleation at similar temperatures (Hill et al., 2016; Morris et al., 2013), and some soil pathogenic bacteria, such as *Pseudomonas syringae*, produce INAs which nucleate water freezing at temperatures close to -2°C (Pearce, 1999; Wisniewski et al., 1999). In addition to these INAs, soils also contain humic and fulvic acids, as well as sterols and membranes coming from decaying root material, which can nucleate ice formation at temperatures as high as -4°C (Hill et al., 2016). Interestingly, Hill et al. (2016) observed that soil contains about 10 000 times more INAs able to initiate ice nucleation at temperatures below -5°C than INAs able to ice nucleation at temperatures above -5°C.

Similar to monocot roots, conifer roots seem to be frost tolerant. Indeed, Coleman et al. (1992) observed that the ice nucleation temperature was several degrees higher than the LT_{50} in the roots of four conifer species, suggesting they were able to withstand intercellular ice formation. Interestingly, it seems that mycorrhiza does not influence root frost hardiness (Korhonen et al., 2018, 2015).

In a study comparing the frost resistance mechanism of 14 Andean dicots found at high altitude, Squeo et al. (1991) observed that their roots could either tolerate or avoid freezing damage. Although the authors note that soil-provided insulation is likely to ensure the survival of most roots, they mention that a frost-avoiding strategy (based on either insulation of supercooling) in such environment could be risky as supercooling and insulation of tissues can only be endured for a few hours.

On the contrary, roots of the *Olea europaea* could rely on a supercooling-based frost avoidance strategy. Using differential thermal analysis, Fiorino and Mancuso (2000) observed that the first and second exotherms (which correspond to the freezing of extracellular and intracellular fluids respectively) of cold-acclimated coarse roots of *Olea europaea* were of -7.4 and -8.1°C. Similar results were observed in the fine roots. This indicates that while the roots of *Olea europaea* seem to have a high frost-avoiding capacity, they die nearly as soon as ice nucleates within their tissues. However, the precise degree to which *Olea europaea* roots are able to supercool their fluid under natural conditions are unknown as all these measures were done using excised and washed roots and therefore, soil INAs were not present.

In addition to these two frost defence strategies, the roots of some perennial monocots have been found to senesce in a fashion similar to how deciduous plants shed their leaves in autumn. In a study on 26 species of perennial monocots, it was found that, depending on the species, the rate of winter survival of their roots was either over 85% or null (Nieman et al., 2018). Interestingly, in plants with a null root survival rate, the roots did not die during the cold season but rather senesced during the cold acclimation period.

Although soils contain water, which can freeze and cause the formation of ice lenses and frost heaves, it seems that the main source of damage to the roots is direct cellular damage rather than mechanical damage (Cleavitt et al., 2008). The main source of cellular damage during freezing stress depends on the strategy adopted to cope with frost. Frost-avoiding tissues are mainly damaged by intracellular ice formation, which leads to cell death. In supercooling-based frost-avoiding plants, as supercooled fluids eventually freeze, the freezing front propagates at a rate of several centimetre per second; the more the liquid is supercooled, the faster the propagation (Pearce and Fuller, 2001). A high propagation speed can lead to freezing of intracellular fluids and cell death. in uprooted *Lolium perenne* and *Poa supine,* Stier et al. (2003) observed that the freezing front propagation was faster in roots than in leaves. However, the presence of INAs in soil and the insulation provided by soil (due to its high specific heat and the release of latent heat during the freezing of soil water) makes that studies on isolated roots may result in artefacts and that in reality ice nucleation in roots is initiated at temperatures close to 0°C. Therefore, ice would propagate a much slower speed than those observed with infrared thermal analysis on isolated roots. Nevertheless, this also indicates that while aboveground organs of monocots contain ice propagation barriers, it is not the case for their roots. Miller and Neuner (unpublished, in Wisniewski et al., 2014) observed that the rhizoderm of seedlings do not have an ice-propagation barrier. This could be different in

mature dicot roots, which have endodermis and exodermis with fully functional Casparian strips and suberized cell walls. These hydrophobic layers are known to act as ice propagation barriers in aboveground organs (Kuprian et al., 2016).

In contrast, in frost-tolerant roots, initial freezing occurs in the extracellular space (which contain INAs and lower solutes levels) at temperatures between -1.5 and -3°C, depending on the cooling speed (Pearce and Fuller, 2001; Stier et al., 2003). When ice nucleates in the extracellular space, its water potential decreases, thereby provoking a net movement of water to the extracellular space. The plasmalemma is the main site of damage of the ensuing intense dehydration mainly through lamellar to hexagonal II phase transition (Strimbeck et al., 2015) and expansion-induced lysis during freeze-thaw cycles (Xin and Browse, 2000). Dehydration also promotes protein denaturation, cytorrhysis and xylem embolism (Charra-Vaskou et al., 2015). It is noteworthy that roots are usually more exposed to frost-induced dehydration damage than aboveground organs. As the soil acts as a thermo-buffer, water in the roots stays liquid for a longer time than in the shoot and can then migrates to frozen extracellular space aboveground, which have a lower osmotic water potential (Bigras and Colombo, 2001; Sakai and Larcher, 2012).

All frost-exposed roots (and organs in general) also face mechanical disruption of their membranes due to the growth of extracellular icicles, as well as organ rupture provoked by ice expanding in their tissues (Burke et al., 1976). In addition to these frost-specific injuries, chilling (exposure non-freezing temperatures) also affects plants in various ways. It promotes membrane phase transition (from liquid ordered to gel-like solid) (Pearce, 2001; Xin and Browse, 2000), increases reactive oxygen species (ROS) production (Aroca et al., 2005; Ruelland et al., 2009), decreases the catalytic activity of enzymatic antioxidants, and it induces the formation of abnormal secondary mRNA structures (Sasaki et al., 2007) (Figure 2).

Frost hardiness

Frost hardiness is organ, species, variety and age-dependent. In woody species, aboveground organs are hardier than roots and there is a frost hardiness gradient from buds (most hardy) to root tips (least hardy), with only a small decrease in frost hardiness at the air-soil interface (Räisänen et al., 2009; Ryyppö et al., 2008; Zhao et al., 1995). Fine roots are less hardy than lignified roots, e.g. *P. sylvestris* roots have an LT_{50} of -4.5°C and -9.1°C, for fine and mature roots respectively (Ryyppö et al., 2008). Surprisingly, Korhonen et al. (2015) observed that the

non-acclimated needles of *P. sylvestris* exposed to various mycorrhizal and fertilization treatments were less frost hardy than the non-acclimated roots (-10.5°C and -8.5°C on average, respectively). In addition, while cold acclimation increased needles frost hardiness up to -14.2°C, the frost hardiness of roots decreased to -9.0°C with cold acclimation. This highlights the difference in response between roots and leaves. Therefore, there is a need to develop new methodologies and techniques specifically designed for studies focused on the root level (Korhonen et al., 2018). Adult plants are hardier than juveniles and seedlings. However, the increase in frost hardiness is far less marked in the roots than in the shoots. Maximum frost hardiness of *Quercus ilex* shoot cambium goes from -16°C during its first year to -25°C when adult (after 3 years), while for roots the maximum frost hardiness goes from -6.5 to -8°C during the same period (Sakai and Larcher, 2012).

In herbaceous species belonging to the Poales order, aboveground organs are also hardier than belowground organs. The hardiest organs are renewal buds and leaf primordia, followed by older leaves and crown and finally roots that are the least resistant to freezing stress (Noshiro and Sakai, 1979; Sakai and Larcher, 2012). Hardened young *Poa pratensis* leaves are resistant up to -15°C, leaf primordia -17°C, winter buds -15°C, rhizome -13°C and fine roots -7°C (Noshiro and Sakai, 1979). Although herbaceous plants can recover from important frost damage to their leaves and roots, damage to their crown (where meristems are found) results in reduced survival rate (Livingston et al., 2016). In herbaceous dicots, roots could play a more important role. For instance, in pot grown *Vicia faba* exposed to freezing temperatures, ultimate survival and shoot production are more closely related to roots survival than to shoot survival (Sallam et al., 2015).

Several hypotheses have been proposed to explain the difference in frost hardiness between roots and shoots. The relative water content is up to 3 times higher in roots than in aboveground organs. This higher water content would be associated with reduced hardiness, presumably because it increases the risk of deleterious ice formation (Bigras et al., 1989; Burke et al., 1976; Levitt, 1980; Pearce, 1999). The insulation provided by soil and the consequent higher fall and winter temperatures to which roots are exposed, could reduce the maximum frost hardiness achievable. Indeed, Magness (1929) observed that *Malus sp*. roots exposed to the air could achieve the same degree of frost hardiness than the shoot. Contrary to this, Ryyppö et al. (2008) reported that *P. sylvestris* roots were less frost hardy than aboveground organs, even when they were exposed to the same temperatures, similar results are reported for horticultural woody species (Pellett, 1971).

Environmental signals inducing cold acclimation and frost hardening

Cold acclimation and frost hardening

In nature, cold acclimation is initiated in late autumn or early winter. This period is characterized by a reduction in day length and lowering temperatures, which both act as environmental signal inducing cold acclimation at the whole plant level. Cold acclimation can also be induced under laboratory conditions, simply by exposing plants to low temperatures (2-8°C) and/or reduced day length. Interestingly, it has been observed that for some species, exposure to cold temperature was enough to induce full acclimation, indicating that cold temperature is the primary cue for cold hardening for these species (Li et al., 2005; Ryyppö et al., 2008; Seppänen et al., 2018).

Different environmental cues induce cold acclimation in aerial and hypogeal organs. In the aboveground organs, cold acclimation can be divided into two or three phases depending on the species. The first phase, usually induced by shortening of the light period, results in growth reduction and the accumulation of starch as a storage resource in the roots (cf. Carbohydrates and sugar alcohols) (Degand et al., 2009; Dumont et al., 2011; Hashimoto and Komatsu, 2007). The second phase, promoted by temperatures close to 0°C, is characterized by the *de novo* production of membrane lipids, proteins and metabolites involved in frost hardiness (cf. Metabolic response) (Beck et al., 2004; Livingston and Henson, 1998). The second hardening phase is usually faster than the first one e.g. Beck *et al*. (2004) reported a hardening rate of 0.3°C per day during the first phase and of 0.9°C per day during the second phase. A third acclimation stage specific to woody species able to resist extremely low temperatures can be triggered by exposure to temperatures between -30 and -50°C. In these plants, water vitrifies inside the cell, which protect them from the fatal consequences of cytoplasm crystallization (Ruelland et al., 2009; Strimbeck et al., 2015).

The root zone temperature seems to be the main environmental signal inducing root hardening (Ryyppö et al., 2008; Sakai and Larcher, 2012). Contrary to most studies, no seasonal impact on root frost hardiness were observed in walnut tree (*Juglans regia*) using electrolyte leakage (Charrier et al., 2013). Aside from the temperature, the photoperiod could also play an indirect role in root cold acclimation via a change in the carbohydrate source-sink relationship between above- and below-ground organs (Smit-Spinks et al., 1985). Two phases of cold-acclimation have been observed in the roots of *J. chinensis* and *P. sylvestris* (Bigras et al., 1989; Ryyppö et al., 2008). The first phase takes place at relatively high temperatures (between 5 and 10°C) and results

in reduced root growth and a slight increase in frost tolerance. During the second phase, promoted by temperatures close to 1°C, root growth totally stops and frost hardiness further increases.

Whether perennial roots enter true dormancy (endodormancy, as defined by Lang et al. (1987)) or remain quiescent (ecodormant) once their growth has stopped has been debated. For some authors, root growth slows down during autumn and roots then enter true dormancy, i.e. their dormancy is controlled by internal factors and they do not immediately resume growth if they are exposed to favourable conditions alone (Johnson-Flanagan and Owens, 1985; Romberger, 1963). For others, roots become quiescent rather than truly dormant: as temperature drops below a certain threshold, roots stop to grow, but they resume growth as soon as favourable conditions arise (Green and Fuchigami, 1985). Since the 80s, no definitive answer has been given (Bigras and Dumais, 2005). Although the knowledge on molecular mechanisms involved in quiescence and dormancy has increased with the advent of cost-effective transcriptomic techniques, we still do not know what are the precise molecular controls that differ between quiescent and dormant plant cells (Considine and Considine, 2016). However, recently Hong et al. (2017) reported that chilling causes DNA damage and induces protective death of columella stem cell daughters. This protective death improved roots ability to overcome cold stress and to resume growth when optimal temperatures were restored.

Deacclimation and dehardening

Temperature is the main environmental factor triggering dehardening of above- and belowground organs (Beck et al., 2004; Ryyppö et al., 2008). Some studies indicate that root dehardening is also affected by the photoperiod (Tinus et al., 2000), although the generalization of this influence is debated (Beck et al., 2004; McKay, 1994). Dehardening might also depend on the duration of the quiescence period (Kalberer et al., 2006; Ögren, 1997). Ögren (1997) observed that during mild winters, Norwegian *P. sylvestris* partially dehardened due to the respiratory consumption of soluble carbohydrates reserves rather than because of the elevated temperature. In general, dehardening is faster than hardening and a significant degree of frost hardiness can be lost in a few hours to days when temperature increases (Cunningham et al., 2003; Kalberer et al., 2006; Taulavuori et al., 2002). For example, while it takes *Deschampsia antarctica* 14 days to gain 5°C of cold hardiness, these 5°C of frost hardiness are lost in 7 days (Chew et al., 2012). Similarly, bilberries (*Vaccinum myrtillus*) exposed to 5°C in mid-winter lost more than 30°C of frost hardiness in 7 days (Taulavuori et al., 2002). Deacclimation dynamics are linked to temperature and plants deharden faster when they are exposed to higher temperatures (Charrier

et al., 2011; Taulavuori et al., 2002). When plants completely deharden, their capacity to subsequently reharden diminishes or can even be lost (Kalberer et al., 2006; Ögren, 1997).

As roots and shoots respond to different environmental stimuli, they do not harden at the same time, i.e. roots begin to cold acclimate later and deharden earlier than aerial parts (Bigras and Colombo, 2001; McKay, 1994). Roots need to stay biologically active for a longer time than shoots to ensure water provision. However, during late winter / early spring warm spells, the root zone temperature can remain lower than the air temperature, which results in limited water supply for aboveground organs and can lead to aboveground dehydration and reduced shoot productivity (Ryyppö et al., 1998). The severity of dehydration stress depends on the delay between the beginning of the warm spell and soil thawing. In an experiment done in growth chambers, Repo *et al*. (2005) observed that exposure of *P. sylvestris* to a warm air temperature and frozen soil resulted in the death of the plants in just two weeks.

Metabolic responses

Molecular perception of cold and signal transduction are required to integrate the environmental signals perceived by the roots and trigger the metabolic responses leading to cold acclimation. These metabolic responses include the accumulation of a variety of osmoprotectants, antifreeze proteins, antioxidants and stabilizing proteins such as chaperones, dehydrins and heat shock proteins (Figure 3). Most studies on cold perception and signal transduction have been done on leaves and seedlings. From these, it appears that the changes of temperature are sensed by the membrane and cytoskeleton, which induce an influx of calcium that triggers downstream responses. Amongst the downstream processes, the signal transduction pathway includes C-repeat binding factors / dehydration responsive element binding factor (*CBFs / DREB*)-dependent and *CBFs*-independent pathways that both lead to the induction of cold responsive (*COR*) genes involved in cold acclimation.

More precisely, plants primarily perceive temperature fluctuations due to changes in membrane fluidity (Plieth et al., 1999). Cold exposure leads to a decreased membrane fluidity and cold acclimation can be artificially induced at 25°C with membrane rigidifying agents while membrane fluidizing agents strongly inhibit cold transduction pathways, even when exposed to 4°C (Furuya et al., 2014; Orvar et al., 2000; Sangwan et al., 2001).

During exposure to low temperatures, the plant cytoskeleton undergoes a rearrangement that is also linked to the induction of genes related to cold acclimation. Treatment of *B. napus* leaves and *A. thaliana* cell suspensions with microtubule and filament stabilizers inhibited the expression of *BN115* and *cas30*, while applying a microtubule and filament dispersant promoted the expression of the same genes (Orvar et al., 2000; Sangwan et al., 2001). Interestingly, Wang et al. (2019) observed that while *Vitis rupestris* microtubules partially disassemble when they are exposed to chilling temperatures (8°C), they totally disassemble when they are cold-shocked (27 to 0°C). In addition, they showed that cells treated with taxol (a microtubule stabilizer) were able to withstand cold shock as well as cells that were cold acclimated at 8°C for 72H.

The increased membrane rigidity and cytoskeleton rearrangement trigger a fast and transient increase in cytosolic free calcium concentration ($[Ca^{2+}]_c$) (cf. Knight and Knight (2012) for an extensive review). Using *in vitro*-grown *Nicotiana plumbaginifolia* expressing aequorin (a calcium-dependent photoprotein used as a $[Ca^{2+}]_c$ reporter), Knight et al. (1991) observed that moving seedlings from 20°C to 5 and 0°C caused a strong transient $[Ca^{2+}]_c$ increase in the whole plant but that exposition to 10, 20 and 40°C did not trigger any change in $[Ca^{2+}]_c$. Using similar experimental setup, Campbell et al. (1996) observed that under gradual cooling (0.1°C.s^{-1}), increase of $[Ca^{2+}]_c$ occurs at 8-10°C higher in roots than in leaves of *N. plumbaginifolia*. This is expected as under natural conditions the roots are buffered against thermal variations by the soil; therefore, any change in temperature could be more significant to the roots than to the aboveground organs. Plieth et al. (1999), who worked with intact root systems isolated from hydroponically-grown *A. thaliana* expressing aequorin, observed that although the magnitude of the $[Ca^{2+}]_c$ elevation mainly depends on the cooling rate, the absolute temperature to which plants are exposed also has an impact. Higher cooling rate increases the magnitude of the cytosolic calcium influx and lower absolute temperatures increase the ability of roots to respond to cold. While often used in the labs to study the response of plants to low temperatures, cold shocks at the root level are unrealistic under natural conditions as the roots are thermo-buffered by the soil. Plieth et al. (1999) could not detect any $[Ca^{2+}]_c$ increase in roots of *A. thaliana* at cooling rate under 0.003°C.s^{-1} (10.8°C.h^{-1}). However, it has been shown in *A. thaliana* cell suspensions that a calcium flux is required for cold acclimation and frost hardening (Monroy et al., 1993). Therefore, it cannot be excluded that the calcium flux is localized closed to the cytosolic face of the plasma membrane or of the tonoplast (Knight and Knight, 2012). The elevation of $[Ca^{2+}]_c$ has been shown to be a main determinant in the induction of *COR* genes (Knight et al., 1996).

In addition to the increase of free cytosolic calcium concentration, a rapid transient increase in phosphatidic acid has also been observed in plant exposed to cold and frost (Arisz et al., 2013; Tan et al., 2018). This increase in phosphatidic acid is generated through diacylglycerol kinase. In barley (*Hordeum vulgare*), the basal activity of diacylglycerol kinase is higher in the leaves than in the roots. However, short term (3h) cold-exposure nearly doubles its activity in the roots but strongly reduces it in the leaves (Peppino Margutti et al., 2018). The phosphatidic acid increase in barley roots has been linked to proline and ROS accumulation (Peppino Margutti et al., 2018). Recently, it has been observed that acyl-coenzyme A:diacylglycerol acyltransferase, which catalyses the conversion of diacylglycerol to triacylglycerol and thereby limit phosphatidic acid production and its possible deleterious effect on the membrane (cf. Cellular membranes), is induced in the roots of *A. thaliana* exposed to frost (Tan et al., 2018). The signal transduction pathway after phosphatidic acid production is not precisely known; however, it seems to imply a *CBF*-independent pathway (Tan et al., 2018).

The calcium signal is then integrated by various calcium/calmodulin binding proteins. In *Arabidopsis* roots, calcium activates a root plasma membrane calcium/calmodulin-regulated receptor-like kinase (CRLK1) (Yang et al., 2010), which in turn activates the MEKK1-MKK2-MPK4 kinase cascade (Furuya et al., 2014). Calcium also activates a calcium sensor-associated protein kinase (CIPK3) (Kim et al., 2003) and other calcium-dependent protein kinases. Eventually, these protein kinase cascades lead to the phosphorylation of ICE1 and ICE2 (inducer of *CBF* expression 1 and 2) which in turn activate the *CBFs* transcription factors (Kim et al., 2015).

CBFs are transcription factors with an AP2/ERF domain, which bind to the C-repeat element. These elements consist of the five base sequence CCGAC and are found in single or multiple repeats in the promoting region of many *COR* genes (Jia et al., 2016; Stockinger et al., 1997). In *A. thaliana*, three *CBFs* transcription factors (*CBFs1-3*) are found in tandem array on chromosome 4. *CBF1* overexpression has been shown to induce *COR* genes and to increase frost-hardiness (Jaglo-Ottosen et al., 1998), while ectopic expression of an apple CBF in peach resulted in increased hardiness and short day induced dormancy (Wisniewski et al., 2011). Detailed functional characterization among others indicates *CBFs1-3* are at least partially redundant (Gilmour et al., 2004; Jia et al., 2016). *CBF2* could have a fine-tuning role in cold acclimation as while it represses *CBF1* and *CBF3* expression (Novillo et al., 2004), an *A. thaliana* ecotypes having a non-functional *CBF2* have reduced cold acclimation capacity compared to ecotypes having functional *CBF2* (Park et al., 2018). Interestingly, although *CBFs* regulate

only around 10% of the *COR* genes, triple *cbfs* mutant lost most of their frost hardening capacity, suggesting a pivotal role of the *CBF*-dependent pathway in frost hardening (Jia et al., 2016; Park et al., 2018).

Once produced, the stability of CBFs proteins is modulated by various effectors. For example, the cold-induced plasma membrane protein CRPK1 (cold-responsive protein kinase 1) can phosphorylate a 14-3-3 protein which downregulates CBFs activity (Liu et al., 2017). Conversely, the cold-induced OST1-phosphorylated BTF3L (basic transcription factor 3 like) protein upregulates CBFs activity (Ding et al., 2018).

There is crosstalk between the signal transduction pathways of different stresses. For example, both the drought and oxidative stress signal transduction pathway imply the MEKK1-MKK2-MPK4 protein kinase cascade. Similarly, abscisic acid (ABA), a hormone traditionally associated with drought stress, can also activate CIPK3 (Kim et al., 2003) and MYB96, a transcription factor upstream of *CBFs* (Lee and Seo, 2015).

Cellular membranes are the primary sites of injury during freezing. One of the first effects of frost injury is an increased leakage of electrolytes from damaged cells to the apoplast (Ryyppö et al., 2008). This makes the measurement of electrolyte leakage one of the primary tools for estimating cold temperature damage and a marker for hardening and dehardening (Pagter et al., 2014). The sources of damage to the membranes are multiple. To begin, ROS can degrade membrane lipids through peroxidation. During lipid peroxidation, a ROS reacts with a hydrogen atom from a fatty acid tail, forming a fatty acid radical. This fatty acid radical can react with other fatty acid tails (therefore propagating the reaction), with itself (forming a cyclic peroxide) or with antioxidants (such as α-tocopherol, glutathione and ascorbic acid; thereby terminating the reaction) (Nayyar and Chander, 2004). Lipid peroxidation mostly affects polyunsaturated fatty acids that possess more reactive hydrogen atoms.

Another effect of lowering temperatures is a shift in the phase of the lipid bilayer, from a liquid-ordered phase to a solid-gel state. The phase transition temperature is function of the number of carbon atoms present in the tail and the number of double bonds (higher unsaturation degree lowers the phase transition temperature). Phase transition reduces water diffusion through the membrane (Eze, 1991) and changes lipid-protein interactions (Iivonen et al., 2004). In order to tackle the reduction in water diffusion, the induction of roots aquaporins (proteins regulating water flux across the membrane) has been observed in roots exposed to cold temperatures (Aroca et al., 2005). An increase in the fatty acid unsaturation has been observed in the roots of

different cold-exposed plant species including *Medicago sativa* (Gerloff et al., 1966), *Hordeum vulgare* (Peppino Margutti et al., 2019), *Brassica napus* (Smolenska and Kuiper, 1977), *P. sylvestris* (Iivonen et al., 2004) and *Glycine max* (Markhart et al., 1980). It is important to emphasize that this change in the fatty acids unsaturation degree is largely due to the formation of new roots rather than the dehydrogenation of fatty acids contained in older roots (Markhart et al., 1980).

In addition, frost also changes the configuration of lipid bilayer. As frost-induced dehydration stress increases, membranes are brought in closer proximity and tend to shift from a lamellar to a hexagonal II phase. Hexagonal II phase membranes are characterized by the formation of inverted cylindrical micelles between the two outermost membranes. This transition is favoured by a higher unsaturation degree of fatty acids, a decreased water content, a higher amount of intracellular membranes, and the presence of phosphatidylethanolamine and sterols in the bilipid membrane (Uemura et al., 1995). Smolenska and Kuiper (1977) observed a reduction in the proportion of phosphatidylcholine (30%) and phosphatidylethanolamine (20%) during the cold acclimation of *B. napus* roots while a 60% increase of an unidentified phospholipid was recorded. Interestingly, while roots of barley contain more phospholipids than leaves, long exposure to cold decreases the phospholipid and glycerolipid content in the roots but not in the leaves (Peppino Margutti et al., 2019). A decrease in free sterols has been observed in cold-acclimated oat leaves while no changes were observed in rye leaves (Laque et al., 2016) and a decrease in the sitosterol (a sterol restricting the motion of fatty acid tails) level has been observed in *P. sylvestris* roots during deacclimation (Iivonen et al., 2004). This transition from a lamellar to the hexagonal II phase may be more frequent in roots as they have a higher sterol content (Rochester et al., 1987), but on the other hand, they also possess less intracellular membranes.

Intense dehydration also provokes expansion-induced lysis. Negative osmotic pressure induces a net movement of water towards the extracellular space and thus reduces cell volume. In the protoplast of non-acclimated cells, this reduction in cell volume provokes the invagination of the plasma membrane and the formation of endocytic vesicles, resulting in a loss of surface of the plasma membrane. Upon rewarming, melted water from the extracellular space goes back to the cell, which causes the burst of the cell before it can regain its former volume. In protoplasts of cold-acclimated cells, the reduction in volume induces the formation of exocytotic extrusions, which does not reduce membrane surface and does not provoke cellular burst upon rewarming (Ruelland et al., 2009; Uemura et al., 1995). The precise mechanism favouring the formation of exocytotic extrusions rather than

endocytic vesicles is unknown. It has been observed that the incorporation of mono- and di-unsaturated fatty acids during cold acclimation promoted exocytotic extrusion (Uemura et al., 1995).

Finally, membranes are also exposed to the destructive effects of extracellular ice crystal formation. The formation of bigger (and more harmful) ice crystals during ice recrystallization is favoured by repeated freeze-thaw cycles (Antikainen et al., 1996; Zachariassen and Kristiansen, 2000) and is tackled by several mechanisms mentioned below.

Plant cell walls are dynamic structures mediating the interaction of plant cells with the environment. Several reports in literature have described an active role of the cell walls in response to abiotic constraints (reviewed by Le Gall *et al*. (2015)). The space between adjacent cell walls is thought to be the main site of ice nucleation as it is where most of the INAs are found (Goldstein and Nobel, 1994). Cell wall plasticity is linked with frost resistance, and in several species cold acclimation has been reported to induce cell wall strengthening (Arias et al., 2015). This strengthening could reduce ice propagation (Sasidharan et al., 2011) and damage to the cell membranes during thawing (Zhang et al., 2016). Some plants also developed strategies to cope with ice propagation, under the form of faults (flexible junctions that can expand and accommodate the formed ice crystals) connecting surface tissues with internal ones and anchorages overlying the vascular bundles (McCully et al., 2004). Furthermore, the anatomy of the conductive system also plays a pivotal role in frost tolerance: in a study analysing Patagonian tree shrubs, species with smaller xylem vessels showed enhanced stem supercooling ability, with ice nucleating at lower temperatures (Zhang et al., 2016). Interestingly, species adapted to lower temperatures had larger xylem vessels and therefore tended to freeze at higher sub-zero temperatures.

Low temperatures also affect cell wall composition. For example, it has been observed that the roots of cold-sensitive chicory (*Cichorium intybus*) varieties exposed to low temperature had lower methylesterase (PME) activity than cold-tolerant varieties (Thonar et al., 2006). Similarly, overexpression of a PME inhibitor was found to reduce freezing tolerance of *A. thaliana* roots (Chen et al., 2018).

Different responses to low temperatures have been reported in gene expression studies: for example, low temperature stress represses genes involved in secondary cell wall biosynthesis and lignification in the roots of alfalfa (*M. sativa*) (Behr et al., 2015). In stems of alfalfa exposed to cold, the genes encoding secondary cell wall

cellulose synthases are downregulated (Guerriero et al., 2014). Leaves of frost-tolerant *Miscanthus* clone showed an increased activity of hydroxycinnamyl alcohol dehydrogenase (CAD) and phenylalanine ammonia lyase (PAL), thereby suggesting shunting carbon towards the synthesis of phenylpropanoids, which however did not result in an increased lignin content (Domon et al., 2013). Species differing in cell wall composition likely show varying cell wall-related strategies to counter low temperature stress, multi-pronged approaches coupling –*omics* with cell wall analysis via monoclonal antibodies and chemical characterization will contribute to shed light on the complex mechanisms involved.

The antioxidant system has to adapt to the unbalance of the redox homeostasis caused by frost stress. Reactive oxygen species such as superoxide anion, hydrogen peroxide and hydroxyl radical are generated by plants as part of their metabolism but exposure to biotic and abiotic stresses lead to an increased accumulation of ROS (Foyer and Noctor, 2009; Ruelland et al., 2009). They have a dual function in plants i.e. they act as secondary messengers in signal transduction at low concentrations (Berni et al., 2018; Foyer and Noctor, 2009) while at higher concentrations they cause oxidative damage ultimately leading to cell death (Rhoads et al., 2006). In roots and other heterotrophic tissues, ROS are mainly produced by the mitochondrial electron transport chain complexes I and III. Complex III releases ROS in the mitochondrial intermembrane space from where they can migrate to the cytosol (Rhoads et al., 2006). Peroxisomes are another ROS source through lipid beta-oxidation although their precise impact on the total ROS production in heterotrophic plant cells is unknown (Miller et al., 2010). Under non-stressing conditions, there is a balance between ROS production and ROS scavenging. However, cold disrupts this balance by inducing the rigidification of mitochondrial membranes (Saha et al., 2015) and provoking the loss of complex IV (Prasad et al., 1994). These events impede electron transfer reactions, leading to increased ROS production. Since cold directly impacts enzymatic activities, including that of proteins contributing to the maintenance of the cellular redox balance, this is exacerbated by freezing temperatures (Baek and Skinner, 2012).

To alleviate the oxidative challenge induced by low temperatures, plants can either reduce ROS production or increase ROS scavenging capacities. The level of ROS produced in the roots during cold stress can be reduced by the induction of the mitochondrial alternative oxidase pathway, alternative NAD(P)H dehydrogenases or uncoupling proteins (Heidarvand et al., 2017). Cold-acclimated plants and cold-resistant cultivars have a higher level of alternative oxidase than non-acclimated plants and cold-sensitive cultivars (Heidarvand et al., 2017).

Several enzymatic and non-enzymatic antioxidants are cold-induced, and their concentrations have been linked to cold hardiness. Table 1 summarizes some of the changes in the activities and concentration of different antioxidants observed in the roots of cold-acclimating plants. In addition, cysteine synthase, an enzyme resulting in the production of cysteine, an amino acid essential in glutathione synthesis, has been found to be induced in cold exposed rice roots (Lee et al., 2009). In an "omics" study comparing root and leaves of cold-acclimating strawberries (*Fragaria* x *ananassa* cv. Korona), Koehler et al. (2015) observed an initial decrease in cysteine in both the roots and the leaves, followed by a subsequent accumulation of cysteine in the roots, but not in the leaves.

Carbohydrates and sugar alcohols are associated with cold acclimation and frost hardiness and have protective effects. (1) They are compatible solutes i.e. low molecular weight soluble molecules that accumulate at high concentration with no cytotoxic effects. As such, they relieve osmotic stress caused by the frost-induced lower water potential of extracellular fluids. (2) They lower the freezing point in a colligative way and appear to be determinant in the water vitrification process (Strimbeck et al., 2015; Wolfe and Bryant, 1999). (3) Sugars (defined here as oligosaccharides) and sugar alcohols stabilize membranes and proteins during intense dehydration. While it was thought that sugar molecules replaced water molecules in the hydration shell of proteins (Xin and Browse, 2000), Di Gioacchino et al. (2018) observed with neutron diffraction that trehalose forms a protective shell that traps water molecules at the surface of proteins, thereby avoiding dehydration-induced denaturation. Sugars seem to protect lipid bilayers from dehydration with a similar mechanism (Konov et al., 2015). By providing a protective shell, sugar molecules limit membrane fusion and protein aggregation by keeping intracellular complexes apart from each other (Mensink et al., 2017). Trehalose and maltose are particularly effective in stabilizing membranes and proteins, and inhibit membrane fusion, even at low concentrations (Wolfe and Bryant, 1999). Glucans also inhibit membrane fusion, with increased capacity as the polymerisation degree increases. In addition to these protective properties, sugars also lower the gel-to-liquid crystalline phase transition temperature of lipid bilayers, hence allowing them to stay functional at lower temperatures. A decrease in phase transition temperature of more than 30°C in liposomes in the presence of sucrose and low molecular weight raffinose family oligosaccharides (RFOs) and of more than 20°C in the presence of fructans and glucans was reported (Hincha et al., 2003, 2002). (4) The accumulation of simple carbohydrates has a predominant role in tolerance to extremely low temperatures in woody plants (under -40°C)

by favouring water vitrification rather than crystallization (Mensink et al., 2017). Trehalose has a higher glass transition temperature and is less prone to crystallization than most carbohydrates, making it particularly important for tolerance to extremely low temperatures (Mensink et al., 2017; Strimbeck et al., 2015). Sucrose tends to crystallize at high concentrations but small amounts of RFOs reduce sucrose's propensity to crystalize (Wolfe and Bryant, 1999). Additionally, fructans interact with ice crystals and change their growth pattern and morphology (Shearman and Olien, 1973). (5) Sugars and sugar alcohols protect the cells from oxidative damage through active ROS scavenging (Keunen et al., 2013). Amongst them, raffinose, galactinol, sorbitol, mannitol and myo-inositol appear to be the most effective free radical scavengers (Folgado et al., 2015; Morsy et al., 2007).

Sugar accumulation is highly correlated with frost hardiness. Hardier plants accumulate sugar earlier and to a higher extent than cold-susceptible plants (Cunningham and Volenec, 1998; Seppänen et al., 2018). During cold acclimation, sugar accumulation takes place in two phases. First, shortening of the photoperiod triggers a change in the allocation of photosynthates from shoot growth to non-structural carbohydrate reserves in the large roots, where it accumulates mainly under the form of starch (Cunningham and Volenec, 1998; Seppänen et al., 2018). This increase in starch concentration is poorly and/or negatively correlated with frost hardiness (Kalberer et al., 2006; Seppänen et al., 2018; Tinus et al., 2000). During the second phase, this starch is hydrolysed into simple sugars, resulting in an increased frost hardiness (Koehler et al., 2015; Lennartsson and Ögren, 2002; Seppänen et al., 2018). The environmental signal triggering starch hydrolysis is not yet deciphered: for long it was thought that lowering temperatures triggered this response, but enzymes involved in starch degradation were already induced before temperatures dropped in some poplar species (Guy et al., 2008).

In alfalfa roots the starch content increases with shortening days and is at its maximum by the end of September when the level of soluble sugar is at its minimum (Cunningham and Volenec, 1998; Seppänen et al., 2018). With lowering temperature, sucrose and RFOs accumulate while starch, glucose and fructose concentrations decrease. Interestingly, cold hardiness is more correlated with the accumulation of RFOs ($r^2 = 0.92$) than with glucose and sucrose ($r^2 = 0.48$) in the roots of alfalfa (Cunningham et al., 2003). Similarly, in poplar roots, frost hardiness is better correlated with trehalose and RFOs content than with glucose, fructose and sucrose concentrations (Regier et al., 2010). Furthermore, *P.* abies and *Carthamus tinctorius* roots also proportionally accumulate more raffinose than sucrose when exposed to chilling temperatures (Landry et al., 2017; Wiemken and Ineichen, 1993). Koehler et al. (2015) observed the accumulation of raffinose, galactinol, and glucose in both

the roots and the leaves of cold-acclimating strawberry, while fructose accumulated only in the roots, and glucose only in the leaves.

Seven genes implicated in trehalose synthesis *AtTPS2*, *AtTPS3*, *AtTPPD* and *AtTPPF* (Iordachescu and Imai, 2008), and *AtTPPA*, *AtTPPG* and *AtTPS11* (Kreps et al., 2002) were reported to be up to 48 fold more expressed in cold-exposed *A. thaliana* roots. Kreps *et al.* (2002) also detected a 65-fold increase of the *AtBAM3* transcript, coding for a β-amylase involved in maltose accumulation; more than 20-fold increase in two galactinol synthase transcripts (involved in RFOs synthesis); and an up to 7-fold increase in β-glucosidase mRNA levels in the roots of cold exposed *A. thaliana*. Galactinol synthase transcripts were found to increase in cold-acclimating alfalfa roots and this increase in transcript level was followed one week later by the increased accumulation of RFOs (Cunningham et al., 2003). Degand *et al.* (2009) observed an important increase in fructan:fructan 1-fructosyl level, involved in fructan synthesis, in cold-acclimated chicory roots.

Polyamines and free amino acids. Polyamines (PAs) are small ubiquitous, polycationic aliphatic molecules, having at least two amino groups. PAs are positively charged at physiological pH and have been shown to bind to negatively charged proteins, acidic phospholipids and nucleic acids, stabilizing them. In non-stressed plants, PAs have a wide array of functions including the regulation of cell proliferation, differentiation, morphogenesis and senescence (Minocha et al., 2014). Increased levels of PAs have been observed in plants exposed to various stresses, including chilling, and is usually associated with increased stress tolerance (Saha et al., 2015; Strimbeck et al., 2015). In addition to their stabilizing properties, PAs have radical scavenging properties (Gupta and Huang, 2014). However, this is debated as Langerbartels *et al.* (1991) observed that while conjugated polyamines have high radical-scavenging activities, radical-scavenging properties of free polyamines are rather poor. Furthermore, anabolism and oxidative catabolism of polyamines were reported to substantially increase H_2O_2 levels (Minocha et al., 2014; Tang and Newton, 2005).

In plants, the most commonly found PAs are putrescine (Put; a diamine), spermidine (Spd; a triamine) and spermine (Spm; a tetraamine) (Minocha et al., 2014). Put is synthesized through decarboxylation of either arginine or ornithine and is used as a substrate to generate higher PAs. Spd and Spm are synthesized through the addition of an aminopropyl residue on Put and Spd, catalysed by Spd and Spm synthase respectively (Lutts et al., 2013). Other PAs have been found in stressed plants such as cadaverine (in *Leguminosae*, *Solanaceae* and

Graminae) (Lutts et al., 2013), cadiamine (in *Leguminosae*) (Smith, 1975), agmatine (Rácz et al., 1996), thermospermine (a spermine isomer) (Saha et al., 2015) and various conjugated polyamines (Guo et al., 2014).

Roots and shoots have different PAs accumulation patterns. Koehler et al. (2015) observed that ornithine accumulates in the roots of strawberries during cold acclimation but not in their leaves, while putrescine accumulates in the leaves but not in the roots. In addition, methionine (a precursor of spermidine and spermine) accumulated in the leaves but not in the roots. Lee et al. (1997) reported an increase in Put, Spd and Spm in cold-exposed *Oryza sativa* shoots while only Put accumulated in the roots. Analogous patterns are observed in the roots of four wheat (*Triticum aestivum*) varieties (Rácz et al., 1996). In addition to the increase in Put content, cold-exposed wheat roots also accumulates agmatine (Rácz et al., 1996).

In a transcriptomic study, Yang et al. (2015) reported that genes involved in Put biosynthesis were cold-induced in both the shoots and roots of chilling tolerant rice varieties, while Spd and Spm related genes were only induced in the shoot. Guo *et al.* (2014) observed no *MfSAMS1* transcript in roots of cold-exposed *M. sativa*. *MfSAMS1* codes for *S*-adenosylmethionine synthetase which catalyses, amongst other, the formation of *S*-adenosylmethionine, the precursor of the aminopropyl residue added on Put and Spd to give Spd and Spm, respectively. Similar changes were reported in the roots and leaves of cold acclimating strawberries (Koehler et al., 2015). Conversely Imai et al., (2004) observed an 8-fold increase in the level of *OsSPDS2* mRNA (coding a putative 42kD spermidine synthase) in *O. sativa* roots exposed four days to low temperatures, and Kreps *et al.* (2002) reported a 2.1-fold increase in the transcript level of a spermine synthase in cold-exposed *A. thaliana* roots. Tang and Newton (2005) observed that while exogenous application Put increased the growth speed of *Pinus virginiana* root tips, Spd and Spm inhibited it.

Some free amino acids are also associated with cold exposure and frost hardiness. They act as compatible solutes and stabilize membranes and proteins in a way similar to sugars. One of the amino acids that has frequently been found to accumulate is proline (Öktem et al., 2008; Yin et al., 2017). In addition to its compatible solute properties, proline also stabilizes polyribosomes and acts as a ROS scavenger and a pH and redox buffer (Hayat et al., 2012). It also limits chilling injury at the whole plant level when exogenously applied to *Vigna radiata* seedlings (Posmyk and Janas, 2007). Interestingly, *Picea asperata* roots, which accumulates high level of proline (typically the pioneer roots) are more resistant to freeze-thaw cycles than roots with increased ROS scavenging capacity (the fibrous roots) (Yin et al., 2017). In addition to proline, alanine and arginine have been found to

accumulate in alfalfa roots (Wilding et al., 1960) and glutamine and glycine betaine accumulation was observed in winter hardy *Beta vulgaris* roots (Loel and Hoffmann, 2015). While the leaves of strawberries accumulate proline during cold acclimation, only a transient increase can be observed in their roots (Koehler et al., 2015).

Antifreeze Proteins (AFPs) are proteins present in different cold-living organisms (plants, insects and arctic fish), that can inhibit and/or curb ice formation (Griffith and Yaish, 2004; Wisniewski and Fuller, 1999). AFPs work in a non-colligative way by binding to the lateral face of ice crystals during ice formation and growth. Typically, crystals formed in a solution containing AFPs are small and form hexagonal columns or hexagonal bipyramids, while in pure water the ice crystals are wider and have a flat disk shape (Griffith and Yaish, 2004; Meyer et al., 1999).

Although fish, insect and plant AFPs have the same name and same mode of action, they differ in structure and protection strategies. Animal AFPs are typically flat and hydrophobic and create thermal hysteresis (difference in temperature between the freezing point and the melting point) of 2 to 6°C (Griffith and Antikainen, 1996). On the other hand, plant AFPs are characterized by multiple highly hydrophilic ice-binding domains in their amino acid sequences containing highly conserved asparagine residues spaced at a regular interval that is complementary to the prism plane of ice (Griffith and Yaish, 2004). Their thermal hysteresis is low (from 0,2 to 0,6°C) but they inhibit ice recrystallization occurring during repeated freeze-thaw cycles or when plants are exposed to low temperatures for an extended period of time (Griffith and Yaish, 2004; Meyer et al., 1999; Wisniewski and Fuller, 1999).

Plant AFPs sequences are homologous to three types of plant pathogenesis-related proteins: endochitinases, endo-β-1,3-glucanases and thaumatins (respectively CLPs, GLPs and TLPs) (Antikainen et al., 1996; Pearce, 2001). This could indicate a double role in cold survival: reduce damage linked to frost and provide defence against psychrophilic pathogens (Griffith and Yaish, 2004). Interestingly, Stressmann *et al.* (2004) found that the chitinase and antifreeze activities of an AFP present in the apoplast of winter rye are tuned depending on the Ca^{2+} concentration. The antifreeze activity was lost when exposed to $CaCl_2$ and recovered when adding a chelator. Conversely, the chitinase activity increased by up to 5-fold in the presence of Ca^{2+} ions.

AFPs are found in the roots of various cold-acclimated mono- and dicotyledonous angiosperms. Antikainen *et al*. (1996) observed AFP specific distribution pattern in *Secale cereale* roots. Three AFPs (a 30 kDa apoplastic CLP,

a 72 kDa intracellular CLP, and a 27kDa intracellular TLP) accumulated in the epidermis of cold-acclimated roots, while only CLPs were found in the endodermis and vascular tissues of non-acclimated roots. An AFP has also been observed in the cold-acclimated taproot of *Daucus carota* (Meyer et al., 1999; Smallwood et al., 1999). This 332 amino acids long protein is, unlike most other AFPs, more closely related to polygalacturonase inhibitor protein than to CLP, GLP & TLP. It is a leucine-rich protein with a glycosylated N-terminal domain and is found in the apoplast. It inhibits ice recrystallization at concentrations as low as 150 µg/ml and its thermal hysteresis is of only 0.34°C (Meyer et al., 1999). More recently, Chew *et al.* (2012) found 60-, 700- and 1000-fold increases in the transcript level of *DaIRIP8*, *DaIRIP1* and *DaIRIP4* (antifreeze proteins) in cold-acclimated *D. antarctica* roots.

Dehydrins (DHNs) are a group of diverse proteins (ranging for 9 to 200 kDa) belonging to the late embryogenesis abundant (LEA) protein group. DHNs are characterized by a repeated C-terminal lysine-rich consensus sequence known as the K-segment (Close, 1997; Hanin et al., 2011; Kosová et al., 2007). Many DHNs also have other conserved segments: the Y-segment (rich in tyrosine) found near the N-terminus and/or the S-segment (rich in serine), which can be phosphorylated as a signal for nuclear targeting (Hanin et al., 2011). Some DHNs also possess a less conserved region rich in hydrophilic residues and glycine called the ϕ-segment (Close, 1997). Close (1997) classified DHNs in five types depending on the segments they possess: K_n, K_nS, SK_n, Y_2K_n and Y_nSK_2. Dehydrins of the SK_n-type are often induced by low temperature (Mingeot et al., 2009). While some DHNs are ubiquitous, other seem to be organ and/or organelle specific (Hanin et al., 2011). The abundance of dehydrins is influenced by the hardening state of the plants, as for instance indicated by the decreased abundance of dehydrins during the sigmoid dehardening process of *Hydrangea paniculata* bark and xylem (Pagter et al., 2014).

In aqueous solution, DHNs adopt a random coil structure due to intermolecular hydrogen bonds with surrounding water molecules and a few intramolecular hydrogen bonds and are therefore classified as intrinsically-disorder proteins (Hanin et al., 2011; Kosová et al., 2007). The K-segment is predicted to form an amphipathic α-helical structure with negative charges at one side, hydrophobic residues at the opposite side and positive charges in between (Hanin et al., 2011; Hara et al., 2017). This K-segment is essential for protective activity as it interacts with the dehydrated surface of other proteins or with membranes and stabilizes them. They also prevent molecular aggregation and protein denaturation and inactivation (Hara et al., 2017). In addition to this stabilizing property, it has been proposed that DHNs act as “space-fillers” and keep cellular complexes at non-harmful distances (Strimbeck et al., 2015). Furthermore, some DHNs have complementary

roles to their stabilizing properties such as ROS scavenging (Hara et al., 2004), metal-binding and transport (Hara et al., 2005), and antifreeze activity (Wisniewski et al., 1999).

Nylander *et al.* (2001) observed four (COR47, ERD14, ERD10/LTI29, LTI30) DHNs in *A. thaliana* roots. With immunohistochemical localization, they showed that ERD10/LTI29 and ERD14 are expressed at a low level and observed the proteins in root tips and root vascular tissues of control plants. Upon low temperature exposure, the expression of ERD10/LTI29, LTI30 and ERD14 was induced and the corresponding proteins were found to accumulate in all root cells although to a higher extent in vascular tissues. This distribution pattern is similar to that of WCOR410, a membrane-binding DHN found in wheat (Danyluk et al., 1998). The precise localization of COR47 could not be determined but its transcript level increased in the roots after 3h cold exposure. COR47 and ERD10/LTI29 were found to be cold specific as they are strongly induced by cold but only marginally by ABA or saline stress. An ERD14-homologue was also observed in cold-acclimated chicory roots along with CAS15, CiDHN1 and CiDHN2, and a 25kDa dehydrin (Degand et al., 2009; Mingeot et al., 2009). Dehydrins also accumulate during winter quiescence and cold acclimation in the roots of woody plants (Kosová et al., 2007).

Other stabilizing proteins such as cold-induced heat shock proteins (HSPs, sometimes called cold shock proteins CSPs) and chaperones play an important role in cold resistance. HSPs are proteins constitutively expressed in all living organisms that are induced under stressful conditions. They were first discovered associated with the response to heat shock (hence their name), but have ever since been associated with other stress responses. The main function of HSPs is the role of molecular chaperones, helping for the folding of proteins and stabilizing their tertiary structure thereby preventing molecular aggregation. However, some HSP have also been linked to signalling protein degradation and targeting, and other have been shown to bind to the 5' untranslated region of some mRNAs, stabilizing them and enhancing their translation (Jacob et al., 2017). Several HSPs are induced in roots exposed to cold. A CSP with RNA-chaperone activity (AtCSP2) found in *A. thaliana* cold-exposed roots (Sasaki et al., 2007) and different HSP70 (a family of HSP of 70kDa), essential for protein folding, assembly and degradation (Jacob et al., 2017), are cold-induced in roots of spinach (Anderson et al., 1994), chicory (Degand et al., 2009), rice (Lee et al., 2009) and pea (Dumont et al., 2011).

Perspectives

Despite an increasing average global temperature, frost damage at the roots level is likely to increase in the near future. This is mainly due to an increased weather variability and a reduction of the snow cover in winter, exposing roots to lower temperatures and to more freeze-thaw cycles. This is predicted to result in decreased agriculture and agro-forestry productivity in temperate and colder climates (Kreyling et al., 2012).

Several methods can be used to mitigate the deleterious effects of freezing temperatures on the roots and consequently on plant primary production. Direct compensation for the loss of snow cover with foam or an artificial cover to insulate the soil is currently done in Northern tree-nurseries (Snyder and de Melo-Abreu, 2005). Priming *via* chemicals or other stressors is another possibility to increase root frost hardiness (Hossain et al., 2018; Posmyk and Janas, 2007). Similarly, incubation of the seeds with growth promoting bacteria has been shown to induce chilling and/or frost tolerance in the roots of young seedlings (Subramanian et al., 2016). However, these two methods can only be implemented at small or medium scale and would need to be repeated every year to keep their protective effects, making them expensive on the long term.

Another solution is the breeding and/or selection of new varieties with improved frost tolerance at the root level. This can be done through two approaches: breeding and genetic engineering. The advantages are that it would be less costly on the long term and can be generally applied. Nevertheless, breeding is time consuming and while plants that are genetically engineered to constitutively overexpress *CBFs* are more frost resistant, they suffer from dwarfism and phenological changes (Artlip et al., 2016; Sanghera et al., 2011). These developmental defects are due to the *CBF*-induced accumulation of DELLA proteins, a family of nuclear growth repressors (Achard et al., 2008). These unwanted pleiotropic effects can potentially be alleviated by simultaneously overexpressing growth-promoting genes or by engineering DELLA genes so that they are not activated by CBFs. Although such gene stacking has already been done to increase drought tolerance while reducing growth penalties, double transformants still have a primary production closer to *DREB1A*/*CBF3* overexpressors than to control plants (Kudo et al., 2017). Similarly, the use of stress-inducible promotor to control *CBFs*-overexpression can also lead to pleiotropic phenotypes in non-stressed plants, although the growth setback is less pronounced than in plants constitutively overexpressing *CBFs* (Morran et al., 2011). Apart from *CBFs*, overexpression of *COR* genes has been done. AFPs overexpression in the roots of spring wheat resulted in increased root frost tolerance (Khanna and Daggard, 2006). However, in a review on the topic of using AFPs to increase frost tolerance at the root or whole plant level, the authors concluded that none of these studies have resulted in a significant level of

protection (Duman and Wisniewski, 2014). To overcome these issues, the use of molecular markers could hasten breeding. Similarly, editing endogenous *CBFs* genes and/or downstream genes into variant sequences coming from more frost tolerant species/varieties could remove the negative impacts of *CBFs*/*COR* overexpression on plants growth and productivity. However, few frost tolerance markers have been validated for the roots and while it is known that cold differently impacts the molecular responses of roots and above ground organs (Koehler et al., 2015; Kreps et al., 2002), the precise impact of cold on the root molecular response is poorly studied.

Future studies will also need to study the interaction existing between stresses. Indeed, under natural conditions, roots are likely exposed to more than one stress at the time (Mittler, 2006). While some stresses, such as heat shock and drought, could potentially strengthen cold acclimation (Hossain, 2018), others have unknown impact on root cold acclimation.

While there is extensive knowledge on the impact of freezing stress on aboveground organs, the mechanisms of root frost tolerance are largely unknown. This makes that progress in the generation of frost tolerant plant crops, at the whole plant or root level, is limited. In order to attain this aim, a concerted effort of fundamental and agricultural research focusing on root frost tolerance is needed.

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Table 1: Changes in the activities and concentration of various enzymes and metabolites involved in the maintenance of the redox homeostasis in the roots of cold-acclimated plants. Abreviations: GSH: glutathione, AsA: ascorbic acid, APX: ascorbate peroxidase, GuPX: guaiacol peroxidase, GlPX: glutathione peroxidase, GSR: glutathione reductase, SOD: superoxide dismutase, CAT: catalase. +++: more than 4-fold increase, ++: between 2- and 4-fold increase, +: less than 2-fold increase, 0: no significant change, -: less than 2 fold decrease

	C. arietinum (Nayyar and Chander, 2004)	*Oriza. sativa* (Kuk et al., 2003)	*M. sativa* (Gerloff et al., 1967)	*M. ciliaris* (Yahia et al., 2016)	*Z. mays* (Prasad et al., 1994)	*C. intybus* (Degand et al., 2009)	*P. banksiana* (Zhao and Blumwald, 1998)	*P. ginseng* (Devi et al., 2012)	*T. aestivum* (Scebba et al., 1999)	*G. max* (Posmyk et al., 2005)	*L. culinaris* (Öktem et al., 2008)
GSH	+						+++				
AsA	++										
APX	+	+				+	++	++			++
GuPX			+	+	++		+	+	+++		
GSR		+					+				
SOD	+	+				+			+++	0	-
CAT	++	++	+		+	+		++	+++	+	0

List of Figures:

Figure 1. Soil freezing patterns expected in the future. In the future, increased temperature could lead to a decreased snow cover leading to a less insulated soil and lower soil temperatures. This would decrease the fine root biomass by up to 50% in summer, highly reducing the new aboveground biomass.

Figure 2. Source of damage caused by low temperatures in plants
The main source of damage during freezing temperatures are intracellular ice formation, which leads to cell death, and extracellular ice formation, which provokes osmotic stress. This osmotic stress mainly causes changes in lipid polymorphism, expansion-induced lysis, which leads to cell death, and an increased ROS production. In light blue, stressors; in red, damage done to the membranes; in yellow, imbalance in ROS homeostasis; in dark blue, alterations in cellular metabolites and macromolecules; in purple, physical damages. Skulls mean direct death of the cell via heavy damage to the membranes.

Figure 3. Protective mechanisms put in place by freezing tolerant roots.
The plasma membrane is the main site of damage during frost-induced stress. During intense osmotic stress, the plasma membrane and the tonoplast can come into close contact and their lipid bilayers can shift from a lamellar to a hexagonal II phase, leading to cellular leakage. The cell wall plays an important role in frost tolerance and its strengthening can lead to increased frost tolerance.
Antifreeze proteins and fructans decrease the expansion of ice crystal in the apoplast and inhibit the formation of icicles. Simple hexoses and free amino acids accumulate in the cytoplasm where they counterbalance the negative osmotic pressure induced by the presence of ice in the extracellular matrix. Dehydrins, heat shock proteins and polyamines stabilize the membranes and inhibit protein and mRNA denaturation. Trehalose, maltose and glucans stabilize the membranes and inhibit membrane fusion. Antioxidant concentrations increase and counters the increased ROS production with the help of proline and ROS-scavenging sugar alcohols i.e. raffinose, galactinol, sorbitol.
I, ice crystal; N, nucleus; ↗ ROS, increased ROS production.