



Review

# Cold Stress in Citrus: A Molecular, Physiological and Biochemical Perspective

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**Abstract:** Due to climate change, we are forced to face new abiotic stress challenges like cold and heat waves that currently result from global warming. Losses due to frost and low temperatures force us to better understand the physiological, hormonal, and molecular mechanisms of response to such stress to face losses, especially in tropical and subtropical crops like citrus fruit, which are well adapted to certain weather conditions. Many of the responses to cold stress that are found are also conserved in citrus. Hence, this review also intends to show the latest work on citrus. In addition to basic research, there is a great need to employ and cultivate new citrus rootstocks to better adapt to environmental conditions.

**Keywords:** citriculture; citrus; rootstock; cold stress



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## 1. Introduction

### 1.1. Abiotic Stress

Climate change is bringing about changes in the weather that cause severe droughts, cold waves, and waterlogging due to excess rainfall, among others. It may seem paradoxical with climate change and global warming to think that cold waves may be caused by the greenhouse effect. Currently, there are many theories on its origin, but the fact is that increasingly extreme changes occur within seasons in relation to water regimes, such as heavy rains after extreme drought periods, or with thermal imbalances, such as winters with an average temperature that is 1–2 °C higher than all-time records, followed by waves of extreme cold [1–4]. Hence the importance of new improvement programs to obtain new crops that are more tolerant to abiotic stresses. Plant breeding programs against abiotic stress are very complex as they involve many genes. To carry them out, first it is necessary to know the crop of interest very well, how the studied stress affects it and what mechanisms of action the plant uses to survive it.

### 1.2. Citriculture and Cold Stress

On a global scale, citrus is one of the fruit culture sectors with the highest production, which exceeded 123 million tons in 2016 (FAOSTAT, October 2019). The citrus market is generally divided into the production of oranges, mandarins (tangerine, mandarin, clementine, satsuma), lemons, and limes. In 2018, the largest producers in the world were Brazil and China with 17 and 10 million tons of oranges, respectively. Spain is in the top 10 world producers (FAOSTAT, October 2019) with a total production of 4 million tons of oranges in 2018. Spain especially stands out for its mandarin production with 2 million tons, is in second place behind China (19 million tons) and is also the world's leading exporter for exporting more than 50% of its production.

The grafting technique in agriculture has become an essential technique for the survival and cultivation of certain annual and arboreal species. This technique, which combines the aerial part and roots of two different species, allows farmers to improve crops against various problems that can arise, such as the distinct biotic and abiotic stresses

present in the growing medium, low productivity at harvest, or simply due to a change in the harvest season to achieve more competitive prices for its harvest.

Grafting generally affects three important plant processes, namely: the use and transport of water and nutrients, the production and transport of hormones, and the large-scale movement of proteins, mRNAs and sRNAs [5]. The rootstock/variety combination results in transmissibility by the rootstock of different qualities that are considered beneficial for the variety. For example, it is known that *Poncirus trifoliata* is one of more tolerant rootstocks to tristeza virus (CTV) which, when grafted with a CTV-sensitive variety, is capable of transmitting tolerance capacity [6,7]. In other words, rootstocks must be able to transmit a quality desired by farmers to a variety and, depending on the selected rootstock/variety combination, these skills will differ [8–11].

These graft-induced benefits are easy to demonstrate from a physiological point of view, but it is important to give an answer to what happens at the molecular level and be able to offer an integrated view of the long-distance communication between the rootstock and variety. Thanks to certain techniques like RNA sequencing, it was demonstrated that many transcripts are capable of translocating between the rootstock and variety, which gives rise to the communication and long-distance control of different characters [12–14]. Knowledge of the transmissibility of the genomes between both plants allows us to understand and address the problems faced today.

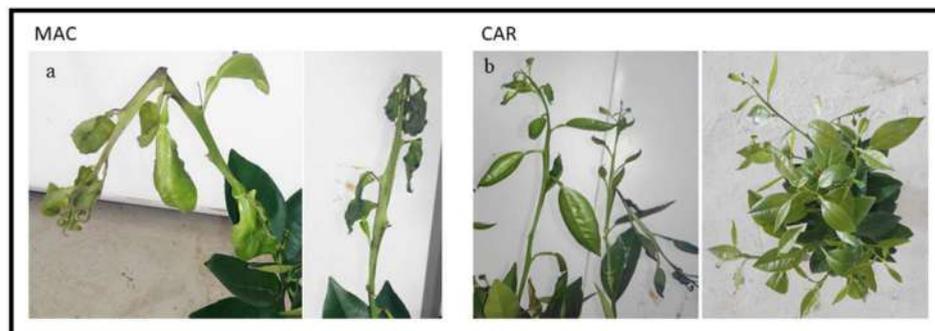
Low-temperature stress is one of the abiotic stresses that leads to most losses in citrus. In December 1990, the losses in California were quantified at 500 million dollars in fresh fruit, and approximately 450,000 ha of affected trees [15]. In Valencia, Spain, cold losses were also very marked in citriculture history. In 2010, two consecutive frosts caused losses of 142 million euros according to AVA-Asaja (Valencian Association of Farmers) Records.

Citrus is considered a tropical and subtropical crop that is generally vulnerable to cold and freezing [16–18]. In Spain, the best-quality citrus fruits are obtained in areas with a temperature range that lies between 23 °C and 34 °C (with a minimum temperature of 13 °C and a maximum one of 39 °C, respectively) [19], with variations between species and varieties. If temperatures lie beyond this range, they may cause massive damage [17]. Citrus fruit are self-inductive crops (i.e., do not need any climatic circumstances to induce flowering) and do not depend on lowering temperatures to flourish [20]. However, flowering and, consequently, production, are markedly stimulated by environmental factors, including a regime of moderately low temperatures under subtropical conditions, and water stress under tropical conditions [21]. The threshold temperature that kills young shoots is –12 °C [22], but some citrus fruit can tolerate a temperature of –10 °C [23] (Figure 1). This is the case of *Poncirus trifoliata* (L.) Raf., which is the most tolerant citrus rootstock to low temperatures [24–26]. Nevertheless, its sensitivity to iron chlorosis is currently in disuse in the Mediterranean region because soil there is extremely calcareous [11,27–29]. Instead, we find that the Carrizo rootstock [*Citrus sinensis* (L.) Osbeck. × *Poncirus trifoliata* (L.) Raf.] and Forner-Alcaide hybrids [28,30–34] are extensively used. Therefore, in addition to the interest that *Poncirus trifoliata* attracts for its resistance, we are also interested in other hybrids grown in the Mediterranean region. The lemon tree *Citrus macrophylla* is used as a rootstock for grafting with lemon trees because it is tolerant to the tristeza virus (CTV). This vigorous rootstock is characterized by its high sensitivity to low temperature [18,24,35,36] and its sensitivity to CTV when it is grafted onto other varieties.

The influence of rootstocks on fruit quality-related traits has a proven significant effect on mandarin fruit size through cell size regulation [37], and also on tree growth, yield and quality, the leaf mineral composition of lemon [38], and even on the flavonoid content of lemon juice [39].

Some sweet oranges [(*Citrus sinensis*) L. Osbeck], such as Tarocco, Moro, and Sanguinello, are characterized by the presence of anthocyanins in both flavedo and pulp, and crops contain the highest flavonoids content in fruit and vegetables [40]. It was shown that the content of these compounds depends on different factors that are both endogenous to the species (pigmented variety, maturation process, etc.) and external or environmental

(exposure to light, nutritional balance, hormones, xenobiotics, and temperature [41]. For these reasons, this variety is very interesting to study low-temperature stress. Table 1 shows the sensitivity/tolerance to cold stress of the different citrus genotypes reported in Florida in Randall Driggers' group [24].



**Figure 1.** Damaged shoots of *Citrus macrophylla* (a) and Carrizo citrange (b) seedlings grafted with the Valencia delta seedless variety exposed to 0 °C for 30 days.

**Table 1.** Citrus cold-tolerant/-sensitive varieties to cold stress [24].

Botanical Name	Common Name	Cold Tolerance
<i>Microcitrus inodora</i>	Large leaf Australian wild lime	Sensitive
<i>Citrus nobilis</i> Lour.	King tangor	Sensitive
<i>C. reticulata</i> ('Clementine' x 'Orlando')	Lee mandarin	Sensitive
<i>Microcitrus australis</i>	Australian round lime	Sensitive
<i>C. reticulata</i> Blanco	Parson's Special	Sensitive
<i>C. sunki</i> hort. ex Tan.	Sunki mandarin	Medium
<i>C. volkameriana</i> / <i>C. limonia</i> Osbeck	Volkamer lemon hybrid	Medium
<i>C. jambhiri</i> Lush.	Florida rough lemon	Medium
<i>C. macrophylla</i> Wester	Alemow	Medium
<i>C. reticulata</i> Blanco	Sun Chu Sha mandarin	Medium
<i>C. sinensis</i> Osbeck	Pineapple sweet orange	Tolerant
<i>C. aurantium</i> L.	Standard sour orange	Tolerant
<i>Glycosmis penthaphylla</i>	Orange berry	Tolerant
<i>C. paradisi</i> 'Duncan' x <i>P. trifoliata</i> )	Swingle citrumelo	Tolerant
<i>Poncirus trifoliata</i>	Citrus trifoliata	Tolerant

Very few studies were carried out to compare grafted citrus under cold stress. A cold hardiness study conducted with a tetraploid Carrizo citrange rootstock showed enhanced natural chilling stress tolerance for common clementine [42]. Carrizo citrange is a widespread rootstock that resulted from crossing sweet orange (*Citrus sinensis* Osb.) and *Poncirus trifoliata* (L. Raf), which possesses a certain degree of the cold tolerance from the *Poncirus trifoliata* parental [43]. Other works with the commercial rootstock Carrizo grafted onto Valencia delta seedless were carried out by María Ángeles Forner and Javier Terol's groups to demonstrate the rootstock influence under cold stress [9,44]. The metabolomic and transcriptomic study performed by these groups indicates that the mechanisms activated in plants shortly after cold exposure remain active in the long term. Both hormone quantification and differential expression analyses suggest that ABA signaling might play a relevant role in promoting the cold hardiness or sensitiveness of Valencia sweet orange grafted onto Carrizo citrange or *Citrus macrophylla* rootstocks, respectively. This work provides new insights into the mechanisms by which rootstocks modulate resistance to abiotic stress in the production variety grafted onto them ([44] under review).

## 2. Cold Stress

Plants are exposed to an environment with its specific conditions and reach an optimal steady state in which their metabolism is optimized for this condition. This includes

adjustments in the concentration and activity of enzymes, and also in the concentration of metabolites, which allow the metabolism to function as efficiently as possible under the conditions it is found. When plants are exposed to a changing environment, a wide range of responses can take place over time. The cellular changes in responses can be divided into three categories.

### 2.1. Molecular Response at Low Temperature

The molecular response at low temperature can occur in two ways:

1. Independent CBF activation pathway (C-repeat binding factors). That is given by the activation of the COR (cold-regulated) response genes from an independent pathway of the CBF transcription factors;
2. Dependent on the CBF-COR route. The CBF regulon is the central response to low temperature. It is an activation cascade that leads to the activation of the COR response genes that depend on the CBF transcription factors.

These COR response genes are rapidly induced from minutes to hours by low temperatures [45]. Many gene products are suggested to be COR genes and act in the acclimatization response and subsequent cold tolerance [46]. These genes include osmoprotector synthesis enzymes, LEA proteins, transcription factors, protein kinases, proteins associated with lipid metabolism, hormone response proteins, cell wall modifiers, and chloroplast proteins [46].

#### 2.1.1. Stress Response by the Dependent CBF Pathway

Sequentially, as stress is detected by plants, membrane destabilization occurs and is the first cause of plant damage by freezing [47,48]. During the acclimatization process, the cryostability process of the plasma membrane increases because of the alteration to the composition of the lipids that alter its behavior when facing dehydration. Uemura's group demonstrated changes in lipid composition during low-temperature acclimatization [49,50]. They specifically observed, on the one hand, an increase in the proportion of phospholipids because of the rising proportion of the di-unsaturated species of phosphatidylcholine and phosphatidylethanolamine and, on the other hand, a smaller proportion of cerebroside lipids [50].

Plasma membrane destabilization leads to a higher concentration of cytosolic calcium [50,51]. Regulation in the proteins that act as calcium sensors is direct or occurs through a signaling cascade from kinases to induce the expression of the genes responsible for the activation of the cold response to pre-existing transcription factors [52–54]. The increase in calcium is transitory and exhibits a characteristic peak caused by a specific vacuolar calcium antiporter known as a calcium exchanger (CAX1  $\text{Ca}^{2+}/\text{H}^{+}$ ) [52] that leads plants to repeatedly react to the low-temperature signal. Thus the second phase activation of the response to cold begins, which prolongs in time in a third phase of response to cold, known as acclimatization [46]. The calcium influx reaches a receptor kinase regulated by calmodulin 1 and 2 (CRLK1 and CRLK2), and begins a signaling cascade of the specific cold pathway (Figure 2) [55,56]. This signaling begins with the activation of protein kinases (MPK) that comprise three protein kinases, MEKK, MKK and MPK, which consecutively act [57,58]. These MPKs can activate or repress the next transcription factor in the signaling pathway, ICE1 (inducing CBF expression 1), which is a MYC-like helix-turn-helix transcription factor that binds in CBF gene promoters [59–62]. These MPKs can activate or suppress upstream of ICE1. Thus, MKK4/5 and MKK3/6 specifically promote ICE1 repression and, consequently, the repression of the response at low downstream temperatures [56,63]. On the contrary, ICE1 expression is induced by MEKK1, MKK1/2, and MPK4, which gives rise to the activation of the cold response pathway. In addition to regulation by MPKs, the stability and, therefore, ICE1 protein activity are also regulated by the post-translational modification of sumoylation by SUMO E3 ligase SIZ1 [62,64,65]. Thus, ICE1 reaches the nucleus and activates the central transcription factors of the cold response: the CBF genes. This pathway of activation of the response genes plays an important role in tolerance to low temperature stress in not only *Arabidopsis thaliana*, but also in other tree

species like poplar, birch, apple, and citrus [25,66,67]. The CBF genes are also known as DREBs (dehydration-responsive element-binding factors) and generally comprise three members, CBF1, CBF2, and CBF3 (also called DREB1b, DREB1c, and DREB1a, respectively), in *Arabidopsis thaliana*. However, this may vary depending on the species. For example, in poplar, the induction of four CBF genes (CBF1, CBF2, CBF3, CBF4) was seen in response to low temperatures [66]. Thus, CBFs activate low-temperature response genes, called COR, which act in both the second phase of the response (minutes–hours) and the acclimatization phase (days–weeks).

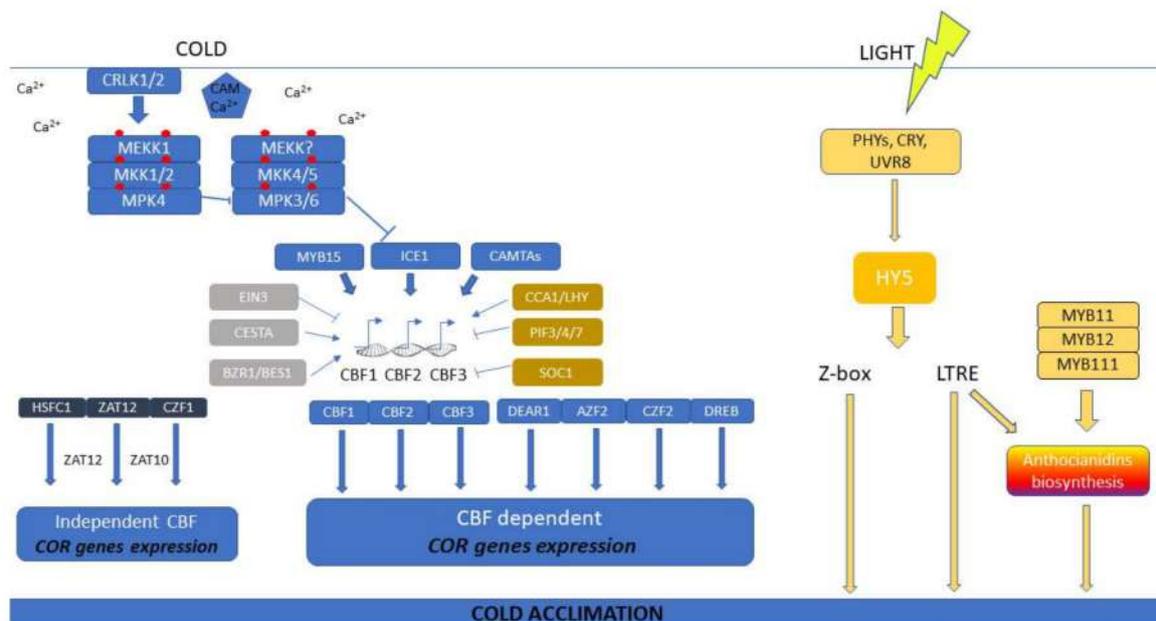


Figure 2. Representation of cold stress signaling, cold genes response, and acclimation.

In addition to ICE1, more activators and repressors of the CBF regulon are known to bind to its promoters and are induced in the presence of cold. MYB15 is an MYB-type transcription factor that can be activated in the presence of low temperatures and binds directly to the promoters of CBF1, CBF2, and CBF3 to bring about their repression [68]. Their expression is also thought to be regulated by MPKs signaling [69,70].

Another transcription factor that activates the expression of CBFs is calmodulin binding transcription activator (CAMTA) [71] (Figure 2). CAMTA 1, CAMTA 2, CAMTA 3, and CAMTA 5 induce CBFs. CAMTA 3 and CAMTA 5 act in the first minutes of temperatures lowering, and it seems to be related to sudden changes in temperature (day/night), and not to progressive changes in temperature which happens with seasonal changes [71,72].

Plants process and integrate the signals surrounding them related to seasonal changes and light. This is a crucial element for their response to acclimatization and tolerance to cold (Figure 2). Phytochromes (PHYA, PHYB, PHYC, PHYD and PHYE) absorb red and far-red light, while cryptochromes (CRY1, CRY2, and CRY3) absorb blue light and UV-A light [73]. Phytochromes are known to activate COR genes like COR15a and COR14b, and regulate the genes targeted by CBFs under near/far-red light conditions [74].

Phytochromes activate the bZIP long hypocotyl 5 (HY5) transcription factor, which happens to be a central modulator of the coordinated response between light signaling and plant development, and also regulates other genes through subnetworks using other regulators [75]. HY5 is overexpressed by low temperatures and mediates the induction of the genes expressed in the presence of this stress due to its binding to the Z-box of promoters, which constitute an LTRE (low-temperature response element) domain or other LTREs yet to be characterized [76].

### 2.1.2. The Molecular Response in Citrus under Cold Stress

The molecular response at low temperatures as indicated above is a general response that it is known in model plants, but previous experiments have found that this response is conserved in *Citrus* spp. [77,78].

In *Citrus sinensis* Osbeck cv. Gannanzao grafted onto the *Poncirus trifoliata* rootstock, CAMTA genes are conserved and intervened in abiotic stress regulation processes, such as cold, salt and drought, and also respond to ABA and JA treatments [3]. Specifically after cold stress treatment, a 3-fold induction of CsCAMTA1, CsCAMTA2, CsCAMTA3, CsCAMTA4, and to a lesser extent of CsCAMTA5, was observed at 24 and 48 h [79].

Another study in *Poncirus trifoliata* showed the conserved function of MAPK in a cDNA amplified fragment length polymorphism (cDNA-AFLP) assay. This group located a putative MAPK3 in *Poncirus trifoliata* and observed that its expression at 4 °C for 17 h was 17-fold vs. its control expression [80].

The inducer of CBF expression gene ICE1, as previously mentioned, has a complex regulation. ICE1 is a bHLH transcription factor and, in citrus, some groups are working to elucidate its function. Liu Jihong's group performed some works in *Poncirus trifoliata* and isolate a bHLB with a probable putative ICE1 function [81–83]. One of these articles shows that PtrbHLH overexpression of tobacco (*Nicotiana tabacum*) or lemon (*Citrus limon*) conferred enhanced tolerance to the cold with chilling or freezing temperatures, whereas the down-regulation of PtrbHLH in trifoliolate orange by RNA interference (RNAi) resulted in marked cold sensitivity [81]. In a second study, the putative ICE1 from *Poncirus trifoliata* overexpressed in lemon and tobacco showed greater activity of antioxidant enzymes, such as superoxide dismutase and catalase, and were detected in transgenic lines under cold conditions. Taken together, these results demonstrate that PtrICE1 plays a positive role in cold tolerance, which may be due to the modulation of polyamine levels by interacting with the ADC gene [82]. A third work in pummel was performed, in which the overexpression of transgenic lines accumulated dramatically lower reactive oxygen species (ROS) levels consistently with marked activity and high expression levels of antioxidant enzymes (genes), including catalase (CAT), peroxidase, and superoxide dismutase [83].

The putative HOS1 gene has been isolated from *Poncirus trifoliata* by Zhong-Hai Sun group in 2010 [84]. In their work, PtHOS1 was cloned, its protein was isolated and its gene expression was quantified in roots, stems, and leaves [84]. Interestingly, the PtrHOS1 expression underwent a declining period in leaves, stems, and roots after cold and ABA treatments, which suggests that the PtrHOS1 expression was down-regulated by both cold and ABA, which occurs in other species [84].

The CBF genes' function was demonstrated in two works in which CBF1 was isolated from citrus and cloned to demonstrate its function when faced with cold stress. The first one, putative CBF1, was isolated from *Poncirus trifoliata* (PtCBF1) and *Citrus paradisi* (CpCBF1) [78]. PtCBF1 accumulated not only earlier, but also at higher levels than CpCBF1. Furthermore, CORc115, a cold-induced group II LEA gene, also accumulated earlier and at higher levels in *P. trifoliata* in response to cold temperatures [78]. The second one was an isolated putative CBF1 from *Poncirus trifotiala*, sweet orange (*Citrus sinensis*), pummelo (*Citrus grandis*), and rough lemon (*Citrus jambhiri*) with complete open reading frames. The results of the multiple sequence alignment and phylogenetic analyses on putative protein sequences suggest that AP2 DNA binding domains and CBF signature sequences are highly conserved in four citrus CBF proteins [85].

The response to cold stress that takes place through light was also conserved in citrus, as demonstrated in two very recent works. One was carried out in pigmented oranges, in which the CgRuby1 promoter was isolated from *Citrus grandis* and overexpressed in *Arabidopsis thaliana* [86]. It was shown how transcription factor HY5 binds to the Ruby1 promoter to give rise to the synthesis of anthocyanins, and this response is also conserved in citrus [86]. Another very recent work about the response to light was carried out by Juan Xu's group, who found that the overexpression of *Citrus sinensis* CsPIF8 led to an increase

in anthocyanin synthesis with low temperatures in transgenic tomato plants and grapefruit calli [87].

## 2.2. Physiological Response

Once signaling occurs, a series of physiological responses takes place in plants. A drop in temperature, without freezing, is harmful for plant tissues because the fluidity in membranes is lost due to the increasing proportion of unsaturated fatty acids that need to adapt to the new conditions.

Changes occur in the structure of chloroplast thylakoids, chlorophyll content, the activity of photosynthetic enzymes and electronic transport, which are associated with a drop in temperatures. However, the relation between physiological changes is not entirely clear [88]. These changes combined with stomatal closure are known to be largely responsible for declining photosynthesis in winter [89].

The result is ion leakage, which is caused by the inactivation of the channels and pumps anchored to the membrane [90], which affect any physiological process that takes place in the membrane that can be cellular and of any other organelle.

### 2.2.1. Membrane Changes in Citrus under Cold Stress

In citrus, changes in the structure of cell membranes and the composition of lipids was also shown as mentioned above. In many studies carried out at low temperatures, losses of ions and ruptures of the membrane occurred, thus damaging the processes taking place there [91–96].

In 1985, George Yelenosky's group analyzed the membrane and damage caused by cold stress in fatty acids. Three genotypes were compared: *Citrus sinensis*, *Poncirus trifoliata* and a hybrid between ((*Citrus paradisi* × *Poncirus trifoliata*) × *Citrus sinensis*). Fatty acids degradation was greater in phosphatidylcholine (89%), phosphatidylglycerol (83%), monogalactosyldiglyceride (79%), and digalactosyldiglyceride (50%). Total leaf fatty acids in the freeze-thaw regime increased 12% in the cold hardened hybrid. Three molecular species of triacylglycerol, which were rich in linolenic acid, increased in the hardened hybrid in the freeze-thaw regime [96]. The increase in highly unsaturated triacylglycerol species under freeze-thaw stress suggests that triacylglycerol plays a role in maintaining the fluidity of biomembranes under freezing conditions [96].

As with *Citrus sinensis* and *Poncirus trifoliata*, for grapefruit (*Citrus paradisi*) it was shown that the expression of the genes involved in both fatty acid biosynthesis and phospholipids degradation was strongly activated, whereas several fatty acid desaturases were down-regulated, except the acyl-[acyl-carrier-protein] desaturase and stearoyl-acyl carrier protein desaturase (Genbank CV706341 and CN182241CV706341CN182241), which were specifically up-regulated in the cold-acclimated fruit [97]. Likewise in blood orange or finger citron, the gene expression of some desaturases lowered [94,95].

### 2.2.2. Photosynthesis and Cold

Photosynthesis is known as one of the most temperature-sensitive physiological processes in plants. Therefore, understanding the physiological processes underlying the temperature response of photosynthesis and its acclimatization is important for both agriculture and the environment. Many plants are well able to adjust their photosynthetic characteristics to their growth needs depending on temperature [98–102].

Some of the mechanisms that lead to low-temperature acclimatization in winter annuals also do so at high irradiation [103–105]. Thus, the photosynthetic adjustment shown by cold-hardened plants resembles that exhibited by plants exposed to high light intensity, while the photosynthetic response of non-hardened plants is imitated by plants growing at low light intensity. The role of light in developing tolerance to freezing is also indicated by improved freezing tolerance under high light conditions with no cold treatment [106]. These results indicate that low temperature and light induce signaling pathways that overlap and accelerate others while plants develop cold tolerance. Several

studies show that light can also substantially broaden the wide range of early protective compounds that are connected during the hardening process [107–109].

#### Photoinhibition

Photoinhibition is a phenomenon that reduces photosynthetic activity, mainly due to the assimilation of the CO<sub>2</sub> induced by excess light [110]. Although reduced photo assimilation may depend on damage to some photosynthetic machinery components, the term inhibition is often used to define the inhibition of photosystem II (PSII) activity [111–113]. As light is the energy required to drive the photosynthesis process, photoinhibition is inevitable when light exceeds the photosynthetic rate. However, the extent of photoinhibition depends on the photodamage-PSII repair balance [114].

Photosystem II (PSII) is the most susceptible component that can be damaged in thylakoid membranes. The main result of abiotic stress is to make PSII prone to photoinhibition [112]. In fact, citrus leaves subjected to temperatures above 0 °C do not show any symptoms. However, when their photosystems are studied by the chlorophyll fluorescence imagine technique (CFI), damage to photosystem II is detected (Figure 3). This technique is very helpful for evaluating damage due to low temperatures. Photosystem I (PSI) is less frequently damaged thanks to the highly efficient photoprotection mechanism, with which it prevents photoinhibition [115]. PSI photoinhibition occurs when PSII's supply of electrons exceeds its ability to accept electrons [116]. When PSI is photodamaged, its recovery process is very long [117]. However, the photoinhibition mechanisms of PSI are not yet well understood.

#### 2.2.3. Photosynthesis and Photoinhibition in Citrus under Cold Stress

In citrus, there are many examples shown in different citrus species that photosynthesis and photosystems are damaged in the presence of low temperatures: in *Citrus junos* [91], in Carrizo citrange [9,42], in Fortune mandarin and Ellendale tangor [118], in *Citrus deliciosa* and *Poncirus trifoliata* var. 'Pomeroy' [119], *Citrus clementina* [42], *Citrus medica* (fingered Citron) and sweet orange [120] and satsuma mandarin [121]. In all cases, photosynthesis (Pn) decreases, stomatal closure takes place (Gs), respiration reduces and internal CO<sub>2</sub> concentration (Ci) rises. Therefore, photoinhibition occurs and stops the function of PSII to lower Fv/Fm values.

#### 2.3. Water Potential

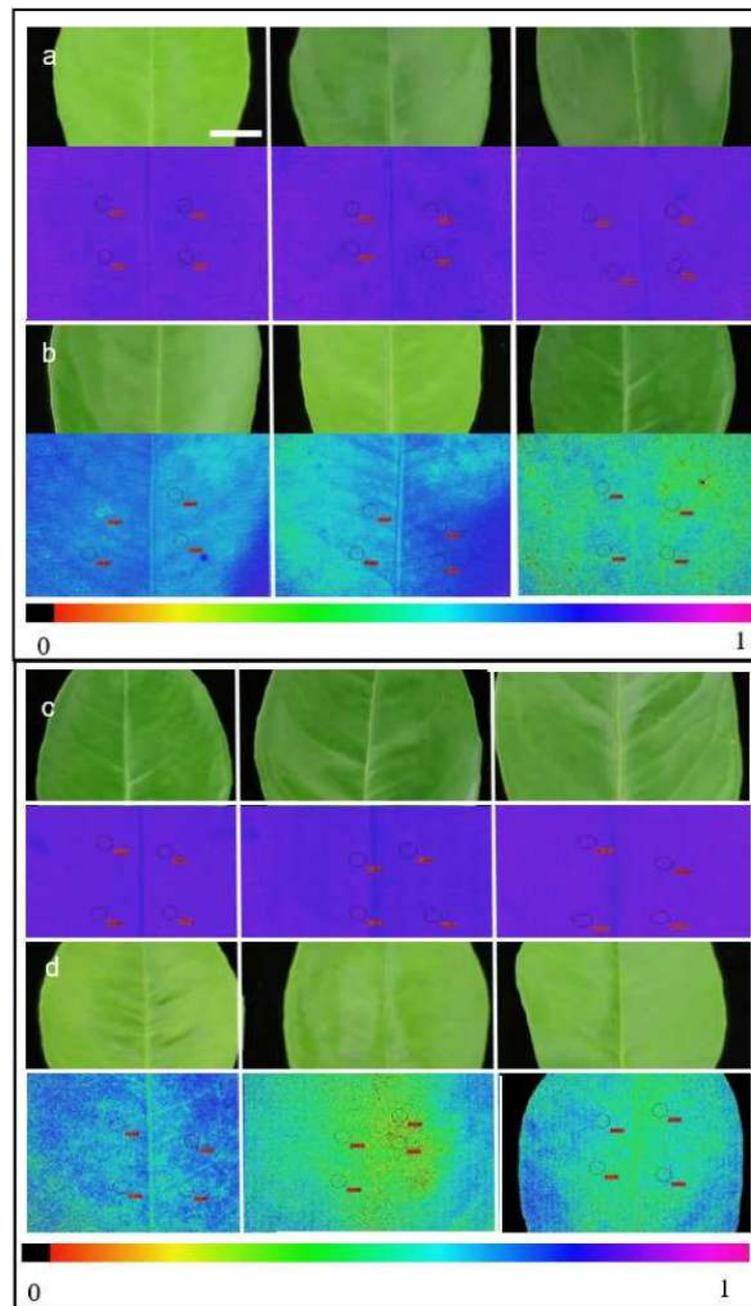
Water balance in plants is very important for physiological, cellular, and metabolic processes to be carried out. Water can be transported in a plant in three ways.

1. Apoplastic transport, which occurs along cell walls and without cell barriers, and gives rise to water transport over long distances that favors the water potential;
2. Transport by the symplastic route, which takes place between cells through plasmodesmata;
3. Transcellular transport through cell membranes. In the presence of abiotic stress like drought, salinity, or temperature stress, plants are compromised between water use to perform physiological CO<sub>2</sub> fixation functions, water photolysis, and transpiration or stopping resources consumption to, thus, stop growth.

#### PIPs and Low-Temperature Stress

Transcellular water transport is carried out mainly by aquaporins. Aquaporins belong to a group of highly conserved membrane-binding proteins, better known as MIP (major intrinsic protein). Aquaporins are a large family of proteins, with 35 genes coding for aquaporins in *Arabidopsis* [122,123], 31 in maize [124], 33 in rice [125], 47 in tomato [126], 55 in poplar [127], 66 in soybean [128], 50 in banana [129], 71 in cotton [130], 41 in sorghum [131], and 34 in sweet orange from [132]. MIPs are classified according to their expression patterns, levels of modification in the amino acid sequence, regulation and intracellular location, which means they are classified into different families. PIPs (plasma membrane intrinsic proteins), NIPs (nodulin-26-like intrinsic proteins) and XIPs (uncategorized X intrinsic

proteins) are generally located in the plasma membrane and are expressed on the cell surface, while TIPs (uncategorized X-proteins intrinsic properties of tonoplast) are located in the tonoplast membrane, which is the vacuole membrane [122]. However, some NIPs and SIPs (small basic intrinsic protein) are located in the endoplasmic reticulum [123].



**Figure 3.** Representation of CFI (chlorophyll fluorescence imaging technique) of cold symptoms in citrus leaves exposed for 30 days to 1 °C. (a,c) are the control groups of Carrizo citrange rootstock and *Citrus macrophylla* rootstock, respectively, grafted with the Valencia delta seedless variety. (b,d) where the cold treatment group of the Carrizo citrange rootstock and the *Citrus macrophylla* rootstock, respectively, grafted with the Valencia delta seedless variety.

#### 2.4. Water Balance in Citrus under Cold Stress

Water balance in citrus in the presence of low temperatures can be contradictory. Low temperatures lead to stomatal closure, decreased transpiration, and a drastically reduced osmotic potential ( $\Psi\pi$ ), which occurs in extreme drought situations in which plants attempt

to not lose water through the transpiration process. However, plants usually have the typical water potential  $\Psi_{\text{stem}}$  and RWC values typical to actively grow, with well-watered trees grown in commercial grove situations on warm sunny days [121,133].

In this case, the transcellular water movement that occurs through aquaporins is very important. Thus, in citrus, from the RNA-seq results obtained in Carrizo citrange and *Citrus macrophylla* grafted onto the Valencia delta seedless variety, it was seen that at low temperature for Carrizo citrange plants that the expression of putative aquaporins PIP1-2, PIP2-2, and PIP2-5 is higher in relation to the low-temperature-sensitive rootstock *Citrus macrophylla* [134], which could be associated with a better use of transcellular water and more tolerance to stress due to low temperatures in the long term, as in other species [129,135,136].

## 2.5. Biochemical Response

As a broad definition, osmoprotectants are small compounds with a neutral electrical charge and low-toxicity organic solutes. They accumulate in large quantities in cells and compensate the imbalance that occurs in the extracellular space when stress occurs. Thanks to their high solubility and them barely interfering with metabolic pathways, they are also called compatible solutes.

### 2.5.1. Osmoprotectors and Low Temperature

Temperate zone plant species acclimatize to the environment in autumn by redirecting their metabolism toward the synthesis of cryoprotective molecules like soluble sugars (sucrose, raffinose, stachyose, trehalose), sugar-alcohol (sorbitol, ribitol, inositol) and low-molecular-weight nitrogenous compounds (proline, glycine, betaine). They also contribute to the synthesis of protein dehydrins, COR and HSPs (heat shock proteins) to stabilize phospholipids, membrane proteins, and cytoplasmic proteins by maintaining hydrophobic interactions and homeostasis, and eliminating ROS. Other solutes are released from the symplast and protect the plasma membrane from ice adhesion and subsequent cell disruption, as discussed above [137–140].

#### Proline

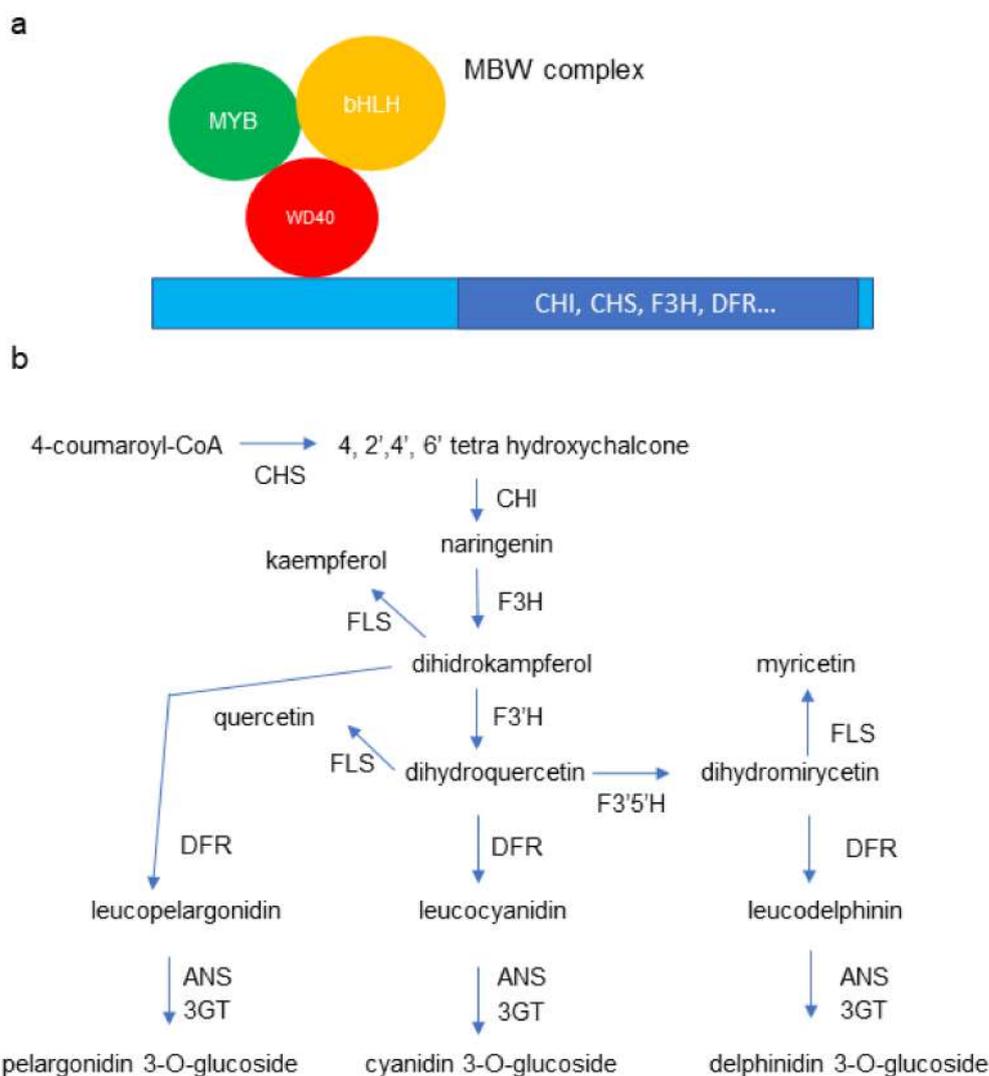
Proline is an amino acid that acts as an osmoprotector. It performs many functions in the cell, but it particularly plays a key role in osmotic adjustment by increasing the ability to resist cell dehydration. Proline is capable of accumulating in some species like citrus [18,141,142] in the presence of certain abiotic stresses, i.e., salinity, drought, waterlogging and temperature stress (heat, cold, freezing) [143–148]. Proline performs its functions at the cellular level by promoting the stabilization of subcellular structures and membranes, the stabilization of proteins upon denaturation, and the detoxification and maintenance of ROS [145,146].

Proline in plants is synthesized mainly from amino acid glutamate (Figure 4), which is reduced in the cytosol to glutamate-5-semialdehyde (GSA) by pyrroline-5-carboxylate synthetases 1 and 2 (P5CS1 and P5SC2) and is spontaneously converted into pyrroline-5-carboxylate (P5C). P5C reductase (P5CR) reduces P5C in proline. However, proline catabolism occurs in mitochondria via proline dehydrogenase (PDH), which produces P5C again, as well as delta 1-pyrroline-5-carboxylate dehydrogenase (P5CDH), which converts P5C into glutamate. Alternatively, proline can also be synthesized by another route from ornithine, which is transaminated in mitochondria by delta ornithine aminotransferase (dOAT) to produce GSA and P5C, which is then converted into proline [143,144,146,149].

#### Anthocyanins

Anthocyanins are not considered to be osmoprotective molecules, but are known to play a key role in some abiotic stresses, such as low temperature, given their properties, such as antioxidant and ROS detoxification [150]. As previously cited, pigmented citrus

varieties turn out to have a high anthocyanin content, which makes them very interesting to study.



**Figure 4.** Schematic representation of the anthocyanin pathway. (a) Sketch of the MBW anthocyanin pathway activation complex and (b) genes that encode their biosynthesis enzymes; CHS (chalcone synthetase), CHI (chalcone isomerase), FLS (flavonol synthetase), F3H (flavonone 3'-hydroxylase), F3'H (flavonoid 3'-hydroxylase), F3'5'H (flavonoid 3'/5' hydroxylase), DFR (dihydroflavonol 4-reductase), ANS (anthocyanidin synthetase), and UFGT or 3GT (UDP-glucose-flavonoid glucosyl transferase).

Anthocyanins are water-soluble pigments from the flavonoid family and are to processes like development, defense and adaptation to unfavorable environments, such as damage from excess UV light and cold [151], as stated above. Anthocyanins are glycosides and acylglycosides of anthocyanidins.

Free anthocyanins are synthesized by the secondary route of flavonoids, and their biosynthesis enzymes are well characterized. Biosynthesis gene activation is regulated by an MBW complex formed by three transcription factors, MYB, bHLH, and WD40, and the MYB transcription factor change depending on the activation gene pathway (Figure 4a) [152,153]. It was demonstrated that the MBW complex is conserved in different species and in active CHI, CHS, F3H, and DFR genes in grapefruit [153] and in PAL, CHS, DFR, ANS, UFGT, and GST in citrus pigmented species [154–156] Biosynthesis begins from amino acid phenylalanine, which becomes a trans-cinnamic acid by the elimination of an

ammonium group by the PAL enzyme (phenylalanine ammonium lyase). In the following steps, four hydroxylations of trans-cinnamic acid take place, which generates *p*-coumaric acid. This is carried out by the C4H enzyme (cinnamate 4 hydroxylase). *p*-coumaric acid is then activated by 4CL (4-coumarate-CoA ligase). The first enzyme in the anthocyanin biosynthesis pathway is CHS (chalcone synthetase), which condenses the malonyl-CoA and *p*-coumaronyl-CoA molecules (Figure 4b) to produce narengin chalcone, which is involved in both the anthocyanin biosynthesis process and the synthesis of phenolic compounds. The narengin chalcone is then isomerized by CHI (for its initials chalcone isomerase) to flavanone narengin, which is then converted into dihydrokaempferol by F3H (flavonone 3'-hydroxylase). Dihydrokaempferol is the starting point of three more compounds, dihydroflavonol, hydroquercetin and dihydromyricetin, which are the result of the reactions catalyzed by F3'H and F3'5'H (flavonoid 3'hydroxylase and flavonoid 3'5'hydroxylase, respectively). Subsequently, dihydroflavonol 4-reductase (DFR) can reduce dihydroflavonols in their respective leucoanthocyanidins (leucopelargonin, leucocyanidin and leucodelphinidin). Finally, anthocyanidin synthetase (ANS) converts leucoanthocyanidins into anthocyanidins, and confers the characteristic color to all three pigments. Once formed (pelargonidin, cyanidin and delphinidin), pigments have to be modified to their active form so that UDP-glucose-flavonoid glucosyl transferase (UGT) catalyzes the addition of glucose to the 3-OH position of anthocyanidins to increase its hydrophobicity and stability [152].

### 2.5.2. Osmoprotectors in Citrus under Cold Stress

In citrus, the increase in the content of osmoprotectors is a preserved response to low temperature stress, with numerous studies in citrus demonstrating this. A relevant work on the synthesis of sugars and starch was conducted in *Citrus junos*. It carried out a proteomics work and bioinformatics analysis, which revealed that most differential abundance protein species (DAPS) participated in starch and sucrose metabolism based on the COG, KEGG, and protein interaction network analyses [91]. For instance, starch synthase (A0A067H6P7), 1,4-alpha-glucan-branching enzyme 1 (A0A2H5PA48), and glucose-1-phosphate adenylyl-transferase (V4S7Z6) were down-accumulated protein species, while a polygalacturonase (A0A067H357) and mannitol dehydrogenase (A0A2H5Q4U0) were up-accumulated protein species under cold stress [91]. Furthermore, the physiological data also demonstrated that the contents of glucose, fructose, and soluble starch significantly increased [91]. A relevant work on the *Poncirus trifoliata* rootstock isolated the PtrBAM1 gene and overexpressed it in tobacco plants. Overexpressing plants leads to increased starch degradation, maltose, and soluble sugars compared to citrus lemon plants [157]. Moreover, the bioinformatics analysis and yeast one-hybrid showed that the PtrBAM1 promoter possessed a recognition zone in the PtrCBF gene [157].

Apart from the proline amino acid conferring tolerance to cold, it has been found to be of much help for selecting tolerant genotypes in citrus [9,92,93,158]. In two works carried out by Abouzar Abouzari, two markers associated with proline content on chromosomes 5 and 8 (CT21 and GT03, respectively) were selected based on the association of the physiological results with molecular markers [92,93].

The anthocyanin synthesis response to cold is also widely known and conserved in citrus fruits, with numerous works showing this fact, where apart from measuring the concentration of anthocyanins and flavonoids, they quantify the gene expression of biosynthesis genes by comparing it among several citrus genotypes [95,159–164]. Angela Roberta Lo Piero's group has analyzed the anthocyanin synthesis route in citrus. One of her works focuses on analyzing the transcriptome of the pigmented citrus variety Tarocco Sciarra [95]. The same work observed the enhancement of the transcripts involved in intense rearranging activity of membrane lipids, mainly to address lipid desaturation by encoding enzymes which form part of both defense mechanisms against oxidative damage and processes that adjust the cell osmotic potential, as well as many ESTs of the genes implicated in primary and secondary metabolisms. In particular, its results

showed that cold stress induces transcriptome modifications, undoubtedly toward the enhancement of both the flavonoid biosynthesis pathways. Those authors propose that the rise in anthocyanin levels that occurs in oranges subjected to cold stress might contribute to control the cell osmotic potential [95].

## 2.6. Hormones and Stress Due to Low Temperatures

### 2.6.1. ABA and Cold

In higher plants, ABA regulates several aspects of plant growth, such as seed development and maturation, germination and stomatal closure [165,166]. The ABA response is necessary for the transcriptional activation of the genes involved in dehydration, low temperature, and other stresses [149,167]. The activation of the ABA response genes leads to the synthesis of the proteins belonging to different families, including the chloroplastic proteins involved in photosynthesis, and in ROS production and detoxification [168]. Other ABA-regulated genes encode transcription factors, chaperones, osmolyte synthesis enzymes, LEA proteins, and COR proteins [169–172].

ABA response is induced by the activation of several protein kinases, including SnRK2s, CPK3, SOS2, CIPK24, and CIPK23 in Arabidopsis, and MdCIPK22 in apple, which affect changes in the phosphorylation of many downstream regulators [60,173–180].

It is known that once the binding between ABA and its PYL receptors takes place, it interacts with type A PP2Cs which, in turn, releases the inhibition of SnRK2.2/3/6 [179–185].

Increased ABA hormone concentration correlates with greater ABA biosynthesis in Arabidopsis and rice [186,187], and occurs in response to cold stress in several species. The exogenous application of the ABA hormone promotes low temperature stress tolerance, and ABA mutants exhibit altered resistance. However, it is thought that ABA does not act at the level of CBF genes expression, but at the upstream control [188–190].

### 2.6.2. Jasmonic Acid and Cold

Jasmonic acid (JA) is a hormone that intervenes in biotic and abiotic stresses that is thought to suppress growth in the presence of cold stress. JA is an oxilipin hormone-type whose concentration increases in cold stress in different plant species, including maize, rice, and Arabidopsis [187,191–193]. A rise in the JA concentration correlates with the increased expression of the biosynthesis genes in Arabidopsis and rice [191,192] and the repression of the genes that code for genes involved in the catabolism of JA in rice [191]. Once the concentration of JA increases, binding occurs with its COI1 receptor and ubiquitination signaling begins. Thus, the proteasome-dependent degradation of JAZ proteins is stimulated, which repress the expression of the JA response genes. Exogenous JA application enhances the induction of the CBFs and CBFs upstream genes after low temperature treatment and promotes cold tolerance. In Arabidopsis, repressors JAZ1 and JAZ4 are thought to interact directly with transcription factors ICE1 and ICE2 to directly repress their effect [192]. A microarray assay in *coi1* mutant plants has shown that some of the COR genes corresponding to the CBF-independent pathway were altered [192].

### 2.6.3. ABA and JAs in Citrus under Cold Stress

Citrus exposed to low temperatures was also affected by ABA and jasmonic hormones, and several studies were carried out in which response to cold is involved [79,84,194,195].

Other studies were also conducted on citrus by applying exogenous ABA, which responded to low temperatures. One study has indicated that foliar ABA applied to citrus effectively increases freeze tolerance if ABA is applied 3 days before the freezing event takes place, but it does not occur when ABA is applied in soil [196]. Subsequently, another ABA application experiment was carried out to compare *Poncirus trifoliata* vs. *Citrus limonia*, and leaves were treated with ABA exposed to low temperatures. Pretreatment with abscisic acid can significantly reduce the membrane damage caused by freezing stress. However, the lipid peroxidation damage caused by low temperature and activities of antioxidant enzymes are irregularly influenced by ABA pretreatment [197].

### 3. Conclusions

This synthesis work of our knowledge about abiotic stress due to low temperatures in citrus fruits was conducted given the need to find answers to this very interesting stress type caused by climate change.

Acquiring further knowledge about rootstocks/variety interactions has allowed us to advance in grafting techniques and to better select plant material from plant breeding programs. The physiological changes caused by low temperatures were known since the 1980s and 1990s [18,21,43]. Stopping photosynthesis, water potential, chlorophyll degradation, and the synthesis of compatible solutes are examples of responses that take place without knowing why. Thanks to the analysis of transcriptomes and genomes, this knowledge sheds light on the lack of knowledge.

In recent years, the most immediate response to low temperatures has become known in model organisms like *Arabidopsis thaliana*. This fact greatly facilitates extrapolating these findings to other horticultural or arboreal species absent in a multitude of loss and gain-of-function mutants.

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