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Mari Iwaya-Inoue · Minoru Sakurai
Matsuo Uemura *Editors*

Survival Strategies in Extreme Cold and Desiccation

Adaptation Mechanisms and Their Applications

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Foreword

The ability of many organisms to survive deep chilling, freezing, and/or dehydration in nature has been known for centuries. However, understanding of the underlying mechanisms by which such organisms escape damage from these environmental insults has until fairly recently (the past 40 years or so) been rudimentary at best. In a now-classic review of the older literature David Keilin in 1959 called this phenomenon "cryptobiosis" or "hidden life" since the organisms in the frozen or dry states showed little or no sign of metabolism or active life. He called life in the dry state "anhydrobiosis" and in the frozen state "cryobiosis". Incidentally, I read this masterful review when I was a 15-year-old schoolboy, and it affected my further career profoundly.

In the 1970s and 1980s a paradigm began to emerge suggesting key roles for small molecules such as polyols and sugars in preserving the integrity of living things in cryobiosis and anhydrobiosis, respectively. Indeed, glycerol in particular and other polyols were implicated as key in frozen organisms and the sugar trehalose in dry organisms. These notions seemed especially attractive since glycerol was shown to be effective in stabilizing cells during freezing; in fact, it became one of the excipients of choice that attained widespread use in cryobiology. Similarly, trehalose was shown to be remarkably effective in preserving membranes and proteins during drying. When the mechanism by which it preserved dry biomaterials was explained in my own laboratory it became widely accepted as a key to survival of anhydrobiosis.

That paradigm dominated thinking until the present century, when it became clear that the mechanisms underlying cryobiosis and anhydrobiosis are more complicated than these single-factor models would suggest. This book summarizes some of what is known of the mechanisms aside from the solute model, and, in some cases, supplementary to it. Several research groups around the world are pursuing work similar to that described here, with promising results appearing from application particularly of techniques of molecular genetics.

Part I of this book deals with cold acclimation, a necessary prerequisite to cryobiosis. Gene regulation during cold acclimation is explored in some detail, both in plants and animals, followed by a contribution on stability of membranes during cold acclimation and effects of cold on microdomains. Other contributions include a study on ice-nucleating proteins in freezing tolerant and sensitive plants. Interestingly enough, freeze-tolerant plants have elevated levels of these proteins, suggesting that they initiate their own

freezing. Particular attention is paid in several chapters to the role of the plant hormone abscisic acid in regulation of responses to cold temperatures.

The second major part is centered on anhydrobiosis, with chapters on specific adaptations in bryophytes and higher plants. Further chapters deal with the role of antioxidants, a synergistic role of proteins and trehalose, and gene-regulatory pathways.

The final part is on applications of findings from cryobiosis and anhydrobiosis. Many such applications are well known in areas such as freezing of gametes for long-term storage. Additional applications are summarized in freeze-drying pharmaceutical products and in preservation of foods.

Finally, I would like to stress again that the emphasis that has been placed on molecules such as glycerol and trehalose as "magic bullets" in stabilizing frozen or dry organisms, respectively, has emerged to be an over-simplification. While these molecules are of no doubt useful for stabilizing biomaterials in vitro, they are not sufficient to preserve intact cells.

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Preface

This present collection of articles on survival strategies for living organisms in extreme cold and desiccation: adaptation mechanisms and their applications had its beginning during the 59th Seminar and Annual Meeting in association with the Japanese Society for Cryobiology and Cryotechnology, held at Kyushu University, 28–29 June 2014. The title of the symposium at the society meeting was "Dormancy in organisms, its role as a survival strategy to adapt against cold/drought stresses". As stated by the first president (1959–1960) of this society, Dr. Ken Yanagisawa, who developed freeze-dried BCG vaccine, "The problem of freezing and drying is related to biology, physics, and other specialized fields. It is believed, both in theoretical and applied study, that progress development is expected for the first time by cooperation of experts in each field."

The subject of the book is to describe how living organisms strategically survive in very severe environmental conditions such as cold and desiccation states and how we can utilize our knowledge gained from them to improve the quality of our life. With this book, we aim to distribute the information we describe as much and as widely as possible to people who are interested in learning from, and who conduct research with, such organisms. To meet this purpose, we have lined up contributors who have been conducting cutting-edge science in related fields. The most important features of this book are (1) a comprehensive description of mechanisms and strategies of living organisms to survive under cold and desiccated conditions at molecular, cellular, and organism levels and (2) revealing the tremendous potential for applications of the findings to a wide variety of industries such as the medical, food, and agricultural and life science industries. For example, basic information on how plants survive under cold, drought, and desiccated conditions may provide a hint on how we can improve crop production in a very fragile environment in global climate change. Studies on cold and desiccation adaptation have revealed unique molecules that protect cells during desiccation such as trehalose and antifreeze protein (AFP), which have a potential use to preserve cells, tissues, and organs for the long term under very stable conditions. In addition, the current progress of supercooling technology of cells may lead us to solve problems related to their high sensitivity to freezing injury, which results in tremendous effects on the usage of these cells. Furthermore, knowledge of water substitution and glass formation as major mechanisms for formulation designs and new drying technologies will contribute to the development of food preservation and drug-delivery systems under dry con-

ditions. We believe that because the progress in the research fields covered by this book has been rapid in the last decade, it is a suitable time to publish this work and provide information to readers effectively with the topics comprehensively covered here.

The editors gratefully acknowledge all authors for their willingness to contribute and thus for encouraging the publishing process. We also appreciate the support from the Japanese Society of Cryobiology and Cryotechnology. Great thanks go to Ms. Misato Kochi, Ms. Yasuko Yamada, and Ms. Chieko Watanabe at Springer, Japan, and Ms. Madona Samuel, project coordinator for Springer Nature, who always were quick to give us their expert advice, invaluable support, and patience during the development of the book. Finally, we thank our colleagues, students, and families, who encouraged us not only during the editing of the book, but also over the long periods of our research.

Fukuoka, Japan — Mari Iwaya-Inoue
Yokohama, Japan — Minoru Sakurai
Morioka, Japan — Matsuo Uemura

Contents

Part I

Adaptation Mechanisms for Cold

1 Gene Regulatory Networks Mediating Cold Acclimation: The CBF Pathway

Javier Barrero-Gil and Julio Salinas

Abstract

Under low nonfreezing temperature conditions, plants from temperate climates undergo physiological and biochemical adjustments that increase their tolerance to freezing temperatures. This response, termed cold acclimation, is largely regulated by changes in gene expression. Molecular and genetic studies have identified a small family of transcription factors, called C-repeat binding factors (CBFs), as key regulators of the transcriptomic rearrangement that leads to cold acclimation. The function of these proteins is tightly controlled, and an inadequate supply of CBF activity may be detrimental to the plant. Accumulated evidence has revealed an extremely intricate network of positive and negative regulators of cold acclimation that coalesce at the level of *CBF* promoters constituting a central hub where multiple internal and external signals are integrated. Moreover, *CBF* expression is also controlled at posttranscriptional and posttranslational levels further refining *CBF* regulation. Recently, natural variation studies in *Arabidopsis* have demonstrated that mutations resulting in changes in *CBF* expression have an adaptive value for wild populations. Intriguingly, CBF genes are also present in plant species that do not cold acclimate, which suggest that they may also have additional functions. For instance, CBFs are required for some cold-related abiotic stress responses. In addition, their involvement in plant development deserves further study. Although more studies are necessary to fully harness CBF biotechnological potential, these transcription factors are meant to be key for a rational design of crops with enhanced tolerance to abiotic stress.

Keywords

Transcription factors · Low temperature · Abiotic stress · Gene regulation · Signaling integration · Hormone signaling · Light signaling

Abbreviations

ABA	Abscisic acid
AP2/ERF	Apetala2/ethylene response factor
BR	Brassinosteroid
CBF	C-repeat binding factor
COR	Cold regulated
CRISPR	Clustered regularly interspaced short palindromic repeats

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CRT	C-repeat
DRE	Dehydration-responsive element
ET	Ethylene
GA	Gibberellin
H3K27me3	Histone H3 lysine 27 trimethylation
ICE	Inducer of CBF expression
JA	Jasmonic acid
RNApol II	RNA polymerase II

1.1 Introduction

Plants often face environmental conditions that are detrimental for their growth and development. Because of their sessile nature that prevents them to escape from adverse situations, many plant species have evolved phenotypic plastic responses that allow them to adjust their physiology to an ever-changing environment. One of the major constraints of plant growth is low temperature, and, not surprisingly, plants from temperate latitudes have evolved a response that allows them to increase their tolerance to freezing. This response, termed cold acclimation (Levitt 1980), is developed after plants are exposed to low nonfreezing temperatures. It comports a variety of structural and metabolic changes including adjustments in membrane composition to prevent damage generated by freezing temperatures, accumulation of stress-related proteins and sugars to avoid the dehydration caused by the immobilization of water around ice nuclei, activation of antioxidant enzymes, and protection of the cold-sensitive photosynthetic machinery (Ruelland et al. 2009; Theocharis et al. 2012). Many years ago, it was found that cold acclimation required changes at the transcriptomic level (Guy et al. 1985). Since then, numerous reports have described that this response involves a comprehensive reprogramming of the transcriptome, modifying the expression of thousands of genes (Hannah et al. 2005; Winfield et al. 2010). Indeed, accumulating evidence indicates that the number and amplitude of the changes in gene expression induced by low temperature correlate positively with the capacity of plants to cold acclimate (Hannah et al. 2006). The study of the molecular mechanisms that govern the expression of cold-regulated (*COR*) genes led to the characterization of a small family of transcription factors, known as C-repeat binding factors (CBFs), which are critical for the accurate development of a cold acclimation response. In this chapter we review the most recent advances concerning the regulation and function of *CBF* genes and discuss the relevance of the CBF signaling pathway to the acquisition of higher freezing tolerance.

1.2 The Identification of the CBFs

The analysis of *cis*-elements in the promoter regions of different *COR* genes from the model plant *Arabidopsis thaliana* allowed the identification of a short sequence of 9 bp, named dehydration-responsive element (DRE), that was able to confer responsiveness to dehydration, salt, and low temperature (Yamaguchi-Shinozaki and Shinozaki 1994). Further analysis enabled to establish a core sequence, CCAGC, called the C-repeat (CRT), which was sufficient for low-temperature induction of these genes (Baker et al. 1994). Using a yeast one-hybrid approach, Stockinger and collaborators isolated an *Arabidopsis* gene encoding a transcription factor with capacity to bind to the CRT/DRE sequence and, thus, was termed C-repeat binding factor 1 (CBF1) (Stockinger et al. 1997). Intriguingly, it was shown that constitutive expression of *CBF1* increased the freezing tolerance of plants, even in the absence of a low-temperature stimulus, and this increase was associated with a rise in the expression of *COR* genes (Jaglo-Ottosen et al. 1998). Soon, it was found that CBF1 was a member of a small family of three transcription factors (CBF1-3) arrayed in tandem (Gilmour et al. 1998; Liu et al. 1998; Medina et al. 1999), all of them having the ability to bind to CRT/DRE boxes in many *COR* genes activating their transcription. These results suggested a relevant role

for CBFs in the development of cold acclimation. The first genetic evidence of CBF involvement in this stress response came with the functional characterization of *cbf2* mutants (Novillo et al. 2004; Alonso-Blanco et al. 2005), showing that disruption of *CBF2* expression altered the capacity of *Arabidopsis* to cold acclimate. Later on, the generation of RNAi lines with knocked-down *CBF1* and *CBF3* expression demonstrated the involvement of all three CBF proteins in cold acclimation (Novillo et al. 2007). However, the degree of redundancy among the *Arabidopsis* CBFs, as well as the relationship among them, has not been clarified until the recent isolation of single, double, and triple *CBF* mutants generated by means of clustered regularly interspaced short palindromic repeats (CRISPR)/Cas technology (Jia et al. 2016; Zhao et al. 2016; Shi et al. 2017). In summary, these studies have revealed that the three CBFs are positive regulators of cold acclimation, controlling the expression of a significant number of genes that mediate the development of this response. The CBFs show a substantial degree of functional redundancy as both *cbf1* and *cbf3* single CRISPR mutants can cold acclimate as a wild type (Jia et al. 2016; Zhao et al. 2016; Shi et al. 2017) and the transcriptome profiling of single mutants displays considerable overlap among the target genes regulated by each CBF (Shi et al. 2017). Since their identification in *Arabidopsis*, *CBF* genes have been also identified in a wide range of plant species in both dicot (Zhang et al. 2004; Benedict et al. 2006; Pennycooke et al. 2008; Welling and Palva 2008) and monocot species (Dubouzet et al. 2003; Xue 2003; Qin et al. 2004; Vágújfalvi et al. 2005).

1.3 The Relevance of CBFs

CBF proteins regulate one of the best characterized cold signaling pathways, but the real relevance of this pathway to the cold acclimation process has only recently begun to be realized. Thus, a transcriptome profiling study of *Arabidopsis* transgenic plants overexpressing CBF transcription factors found that 11% of all *COR* genes identified were controlled by, at least, one CBF (Park et al. 2015). A similar figure was obtained when analyzing an *Arabidopsis* CRISPR mutant devoid of any *CBF* expression, which shows altered expression patterns for about 10% (414) of all *COR* genes in that work (Zhao et al. 2016). Importantly, this alteration results in a severely reduced cold acclimation capacity for this triple mutant (Zhao et al. 2016). An independent study also found that abrogation of *CBF* expression in *Arabidopsis* resulted in defective cold upregulation of *COR* genes with profound consequences in the capacity to cold acclimate (Jia et al. 2016). Nonetheless, both studies revealed that triple *cbf* mutants still retained some capacity to cold acclimate. All these results manifest that CBFs control a significant portion of the *COR* gene expression.

Further evidence on the relevance of the CBF pathway in cold acclimation has been provided through the study of natural variation for freezing tolerance in *Arabidopsis*. Natural populations of *Arabidopsis* are found in a wide range of climates, and substantial diversity in freezing tolerance among different accessions has been reported (Hannah et al. 2006; Zhen and Ungerer 2008). Intriguingly, several papers have described mutations that result in changes in *CBF* expression as a source of natural variation in this species for cold acclimation capacity (Alonso-Blanco et al. 2005; Kang et al. 2013; Oakley et al. 2014; Gehan et al. 2015; Monroe et al. 2016). It is worth mentioning that some of these studies also concluded that, while having fully functional CBF proteins has an adaptive value in cold climates, it provokes deleterious effects in warmer climates where freezing temperatures are uncommon (Oakley et al. 2014; Gehan et al. 2015; Monroe et al. 2016). In addition, CBF function has been found to significantly contribute to winter hardiness in temperate cereals. In both barley andwheat, variation in copy number of *CBF* genes can be associated with tolerance to freezing temperatures (Knox et al. 2010; Würschum et al. 2017). Taken together, all these findings establish that CBF proteins are major contributors to freezing tolerance in cold-acclimating species.

1.4 The *CBF* Promoters Constitute Central Hubs That Integrate Multiple External and Internal Signals

Overexpression of *CBF* genes, besides increasing freezing tolerance, also results in growth reduction, producing dwarf plants that flower later and have a prostrate growth habit (Gilmour et al. 2004). Interestingly, CBF loss of function results in a similar phenotype (Jia et al. 2016; Zhao et al. 2016), suggesting that any deviation from the appropriate level of *CBF* expression is detrimental for the plant. This is further supported by the observation mentioned above that a functional CBF pathway has deleterious effects on warm climates where freezing temperatures are uncommon (Oakley et al. 2014; Gehan et al. 2015; Monroe et al. 2016). It is not surprising, therefore, that *CBF* expression is tightly regulated (Barrero-Gil and Salinas 2017). *CBF* genes are expressed at low levels in warm conditions, but their expression increases transiently by several orders of magnitude within minutes of cold exposure, returning to resting levels after a few hours at low temperature (Gilmour et al. 1998; Liu et al. 1998; Medina et al. 1999). The expression of *CBF* genes has been the object of intense research in the past two decades revealing an intricate network of different transcription factors involved in their regulation (Table 1.1; Fig. 1.1). From these results, a fascinating picture emerges where *CBF* promoters constitute central hubs that integrate not only information about temperature but multiple signaling pathways from both external and internal cues. This section below describes the different signaling pathways that converge on the regulation of *CBF* transcription.

The CBF promoters mediate low-temperature signaling through a beta helix-loop-helix (bHLH) transcription factor called INDUCER OF CBF EXPRESSION1 (ICE1). The existence of this factor was postulated to explain the rapidity with which *CBF* genes are upregulated by low temperatures, anticipating that there should be a factor already present at warm temperatures that would be activated by cold to trigger the induction of *CBFs* (Gilmour et al. 1998). A genetic screen designed to identify such a regulator identified ICE1 as a factor that bound to an E-box element in the *CBF3* promoter inducing its expression (Chinnusamy et al. 2003). ICE1 is activated by cold to relieve the *CBF* repression mediated by the factor MYELOBLASTOSIS15 (MYB15), which binds to the MYB sequences in the promoters of *CBFs* to restrain their expression (Chinnusamy et al. 2003; Agarwal et al. 2006). Recently, it has been demonstrated that ICE1 and its close homolog ICE2 can bind to E-box elements in the promoter region of all *CBFs* to activate their expression (Kim et al. 2015). The CALMODULIN-BINDING TRANSCRIPTIONAL ACTIVATORS (CAMTA) represent another group of transcription factors that, under cold conditions, bind to the promoters of *CBF1* and *CBF2*, in particular to their CGCG motifs, to induce their expression and transduce the cold stimulus (Doherty et al. 2009; Kidokoro et al. 2017).

In addition to low-temperature signals, *CBF* promoters also integrate environmental information about light quality, daylight length, and internal signals from the circadian clock. In this context, the photoreceptor PHYTOCHROME B (PHYB) determines the photoperiodic regulation of *CBF* transcript levels most likely through its interaction with PHYTOCHROME-INTERACTING FACTOR4 (PIF4) and PIF7, which bind to the G- and E-boxes in the *CBF* promoters to repress their expression (Lee and Thomashow 2012). In a similar manner, both PHYB and PHYD perceive changes in light quality and modulate *CBF* expression, which is considered a mechanism that anticipates temperature seasonal changes (Franklin and Whitelam 2007). Furthermore, the expression of *CBFs* is also regulated by the circadian clock in a PIF-independent manner. Some years ago, it was shown that the cold induction of *CBFs* is gated by the clock so that it is much higher if the cold stimulus occurs at ZT4 than at ZT16 (Fowler et al. 2005). Remarkably, the gating coincides with high levels of the circadian oscillator component CIRCADIAN CLOCK-ASSOCIATED1 (CCA1) (Alabadi et al. 2001), which binds directly to the *CBF* promoters and activates cold acclimation

Table 1.1 Transcription factors that directly regulate CBF expression. Different *cis*-elements present in the promoter sequences of CBF genes enable the binding of a collection of transcription factors with positive and negative effects on their transcription

Transcription factor	Signaling pathway	Promoter binding			Techniques	*cis-element*	Output	References
		CBF1	*CBF2*	*CBF3*				
ICE1	Cold	+	+	+	CHIP, EMSA	E-box	Positive regulation of CBF1, CBF2, and CBF3 expression Positive regulation of CBF1, CBF2, and CBF3 expression	Kim et al. (2015)
ICE2	Cold	+	+	+	CHIP	E-box	Positive regulation of CBF1, CBF2, and CBF3 expression Positive regulation of CBF1, CBF2, and CBF3 expression	Kim et al. (2015)
MYB15	Cold	+	+	+	EMSA	MYB	Negative regulation of CBF1, CBF2, and CBF3 expression Negative regulation of CBF1, CBF2, and CBF3 expression	Agarwal et al. (2006)
CAMTA3	Cold	+	+	n.d.	EMSA	CGCG-box	Positive regulation of CBF1 and CBF2 expression Positive regulation of CBF1 and CBF2 expression	Doherty et al. (2009) and Kidokoro et al. (2017)
CAMTA5	Cold	+	+	n.d.	EMSA	CGCG-box	Positive regulation of CBF1 and CBF2 expression Positive regulation of CBF1 and CBF2 expression	Doherty et al. (2009) and Kidokoro et al. (2017)
BZR1	BR	+	+	n.d.	CHIP, EMSA	E-box and BRRE	Positive regulation of CBF1, CBF2, and CBF3 expression Positive regulation of CBF1, CBF2, and CBF3 expression	Li et al. (2017)
CES	BR	+	+	+	CHIP, EMSA	G-box and E-box	Positive regulation of CBF1, CBF2, and CBF3 expression Positive regulation of CBF1, CBF2, and CBF3 expression	Eremina et al. (2016b)
EIN3	ET	+	+	+	CHIP, EMSA	n.d.	Negative regulation of CBF1, CBF2, and CBF3 expression Negative regulation of CBF1, CBF2, and CBF3 expression	Shi et al. (2012)

(continued)

Table 1.1 (continued)

Transcription factor	Signaling pathway	Promoter binding			Techniques	*cis-element*	Output	References
		CBF1	*CBF2*	*CBF3*				
PIF3	ET, light	+	+	+	CHIP, EMSA	G-box and E-box	Negative regulation of CBF1, CBF2, and CBF3 expression Negative regulation of CBF1, CBF2, and CBF3 expression	Jiang et al. (2017)
PIF4	Light	+	+	+	CHIP, EMSA	G-box and E-box	Negative regulation of CBF1, CBF2, and CBF3 expression Negative regulation of CBF1, CBF2, and CBF3 expression	Lee and Thomashow (2012)
PIF7	Light	+	+	+	CHIP, EMSA	G-box and E-box	Negative regulation of CBF1, CBF2, and CBF3 expression Negative regulation of CBF1, CBF2, and CBF3 expression	Kidokoro et al. (2009) and Lee and Thomashow (2012)
CCA1	Circadian	+	+	+	CHIP	EE and CBS	Positive regulation of CBF1, CBF2, and CBF3 expression Positive regulation of CBF1, CBF2, and CBF3 expression	Dong et al. (2011)
TOC1	Circadian	–	–	+	CHIP	T1ME, G-box and EE	Negative regulation of CBF1, CBF2, and CBF3 expression Negative regulation of CBF1, CBF2, and CBF3 expression	Keily et al. (2013)
PRR5	Circadian	+	+	+	CHIP-seq	T1ME and G-box	Negative regulation of CBF1, CBF2, and CBF3 expression Negative regulation of CBF1, CBF2, and CBF3 expression	Nakamichi et al. (2009, 2012)
PRR7	Circadian	+	+	n.d.	CHIP	T1ME and G-box	Negative regulation of CBF1, CBF2, and CBF3 expression	Nakamichi et al. (2009, 2012)
PRR9	Circadian	+	+	n.d.	CHIP	T1ME and G-box	Negative regulation of CBF1, CBF2, and CBF3 expression	Nakamichi et al. (2009, 2012)
SOC1	Flowering	+	+	+	CHIP	CArG	Negative regulation of CBF1, CBF2, and CBF3 expression	Seo et al. (2009)

A plus sign indicates the existence of experimental evidence for direct interaction of a transcription factor to a CBF promoter, whereas a negative sign indicates a failure to find support for such binding. CHIP (chromatin immunoprecipitation). EMSA (electrophoretic mobility shift assay). The effect of the binding of each transcription factor on CBF expression is indicated in the output column

(Dong et al. 2011). Other components of the circadian clock also regulate *CBF* expression negatively, including PSEUDO-RESPONSE REGULATOR5 (PRR5), PRR7, PRR9, and TIMING OF CONSTANS1 (TOC1) (Nakamichi et al. 2009, 2012; Keily et al. 2013).

The *CBF* promoters have also the ability to respond to endogenous signals from multiple hormonal pathways (Shi et al. 2015; Eremina et al. 2016a). For instance, the brassinosteroid signaling transcription factors BRASSINAZOLE RESISTANT1 (BZR1) and CESTA (CES) mediate the activation of the CBFs pathway and, consequently, of cold acclimation (Eremina et al. 2016b; Li et al. 2017). Although further study is necessary to elucidate the precise role played by brassinosteroids (BRs) during the cold response (Barrero-Gil and Salinas 2017), compelling data show that transcription factors BRZ1 and CES can directly bind to CBF promoters and activate their transcription (Eremina et al. 2016b; Li et al. 2017). These findings strongly suggest that *CBF* transcription is determined by BR levels. The levels of jasmonic acid (JA) also play a positive role on the CBF pathway. Indeed, JA accumulates early during the development of cold acclimation, relieving the repression of ICE1 by the JASMONATE ZIM-DOMAIN1 (JAZ1) repressor and allowing the induction of *CBF* expression (Hu et al. 2013). The gaseous phytohormone ethylene (ET), on the contrary, functions as a negative regulator of the CBF pathway and of cold acclimation. Despite conflicting reports about the accumulation of ET in response to low temperature, which may be ascribed to different experimental conditions (Shi et al. 2012; Catalá et al. 2014), conclusive evidence has been presented that cold fosters the disappearance of ET-signaling negative regulators EIN3-BINDING F-BOX 1 (EBF1) and EBF2 (Jiang et al. 2017). This, in turn, results in the accumulation of ETHYLENE INSENSITIVE3 (EIN3) and PIF3 transcription factors that would bind to the *CBF* promoters to repress their expression (Shi et al. 2012; Jiang et al. 2017) (Fig. 1.1).

Low temperature reduces the levels of bioactive gibberellins (GAs) in a CBF-dependent manner, which results in an increase of DELLA proteins that promote cold acclimation (Achard et al. 2008; Richter et al. 2013). The precise mechanism whereby DELLA proteins enhance this stress response remains to be elucidated, but it may be related to their capacity to regulate *CBF* expression since the accumulation of DELLA proteins generates an increase in *CBF* transcripts (Richter et al. 2013). This increase requires the function of two GATA transcription factors, GATA NITRATE-INDUCIBLE CARBON-METABOLISM INVOLVED (GNC) and GNC-LIKE (GNL), which repress the transcription of *SUPPRESSOR OF CONSTANS1 (SOC1)* (Richter et al. 2013). This negative regulation has important consequences for *CBF* expression and cold acclimation as it attenuates freezing tolerance either by antagonizing the ICE1 transcription factor (Lee et al. 2015) or repressing *CBF* transcription through direct binding to *CBF* promoters (Seo et al. 2009). In addition, crosstalk with other hormone signaling pathways may account for the implication of DELLA proteins in the development of cold acclimation. For instance, DELLAs interact with the brassinosteroid mediator BZR1 to repress its transcriptional activity and promote photomorphogenesis (Gallego-Bartolomé et al. 2012). Likewise, DELLA proteins modulate JA signaling through their interaction with the JAZ negative regulators in several physiological processes, including defense against pathogens (Hou et al. 2010). DELLA proteins have also been shown to interact with the ET-signaling mediator EIN3, antagonizing its transcriptional activity during apical hook development (An et al. 2012). Finally, DELLA proteins interact with PIFs to repress growth during photomorphogenesis (de Lucas et al. 2008). Since most of these DELLA interactors are involved in the establishment of cold acclimation response through their capacity to directly bind to *CBF* promoters regulating their expression (Lee and Thomashow 2012; Shi et al. 2012; Hu et al. 2013; Jiang et al. 2017; Li et al. 2017), it is tempting to speculate that DELLAs might modulate the function of CBFs in cold response as well.

Beside BRs, JA, ET, and GAs, it is likely that *CBF* promoters also respond to signals from additional hormones. In this regard, although the cold induction of the *CBFs* was originally defined as independent of abscisic acid (ABA), new data is challenging this notion. First, *CBF* gene expression is induced by exogenous application of ABA (Knight et al. 2004). Second, the induc-

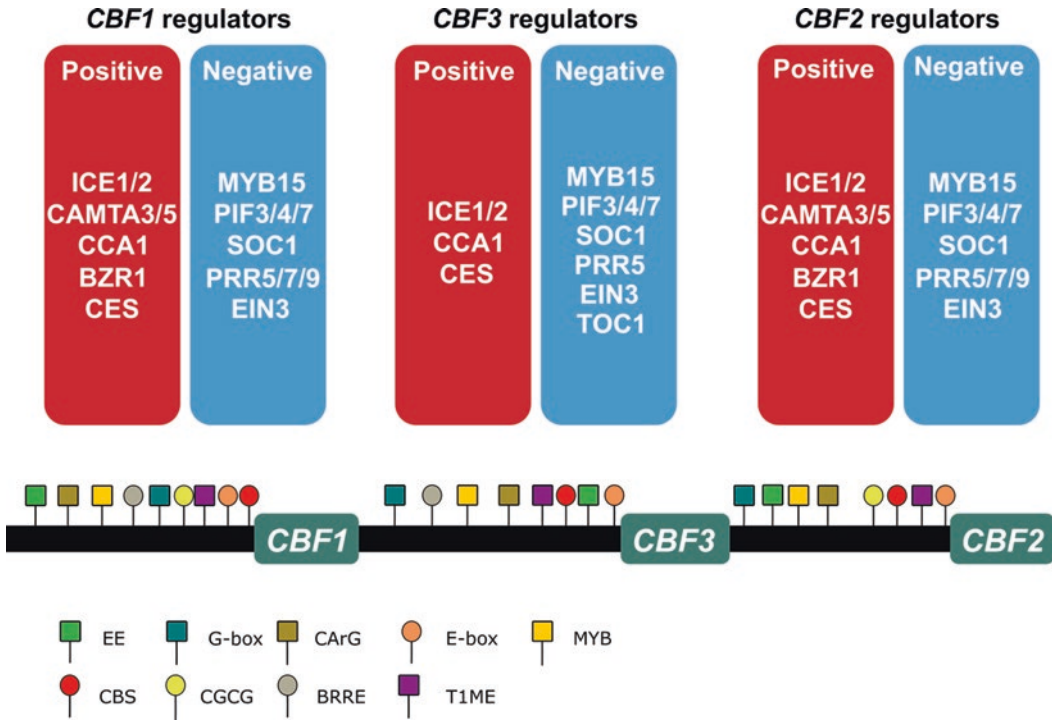

Fig. 1.1 *cis*- and *trans*-acting elements involved in the regulation of *CBF* expression. Different *cis*-elements present in the promoter sequences of *CBF* genes enable the binding of a collection of transcription factors with positive and negative effects on *CBF* transcription. The combinatorial effect of the binding of multiple transcription factors is expected to determine the precise level of expression for each *CBF* gene. An additional degree of complexity is provided by the capacity of many of these transcription factors to interact with other factors with synergistic or antagonistic effects. These interactions have been omitted for simplicity. EE (evening element; AAAATATCT), G-box (CACGTG), CArG box (C[AT]6 G), E-box (CANNTG), MYB (TT/GGTTA), CBS (CCA1 binding site; AATCT), CGCG-box (CGCG), BRRE (brassinosteroid response element; CGTGT/CG), T1ME (TOC1 morning element; TGTG)

tion of *CBF* genes in response to low temperature is reduced in an ABA-insensitive mutant (Ding et al. 2015). Third, several mediators of ABA signaling, including HEPTAHELICAL PROTEIN1 (HHP1), HHP2, HHP3, and OPEN STOMATA1 (OST1), positively regulate *CBF* gene expression under cold conditions by acting on upstream regulators of the CBF pathway ICE1, ICE2, and CAMTA3 (Ding et al. 2015; Lee and Seo 2015) (Fig. 1.1). However, neither the kinase activity of OST1 nor the cold-induced *CBF* expression appears to be altered in *Arabidopsis* plants with reduced levels of ABA (Ding et al. 2015; Lee and Seo 2015). It has been argued that, perhaps, some components of the ABA-signaling pathway may have an ABA-independent role during the development of cold response (Ding et al. 2015). Nonetheless, ABA levels have been shown to increase transiently during cold response (Lang et al. 1994), and the cold induction of some *CBF*-target genes is reduced in ABA-deficient mutants (Nordin et al. 1993; Xiong et al. 2001; Cuevas et al. 2008). Thus, further research is necessary to determine the effect of ABA in the elicitation of the CBF pathway.

What the study of *CBF* expression is revealing is an intricate network of regulatory events, where, in addition to cold signaling, hormone and light signaling pathways coalesce at the promoters of *CBFs* to tightly control their expression. The numerous negative regulatory loops in this network further support the assumption that

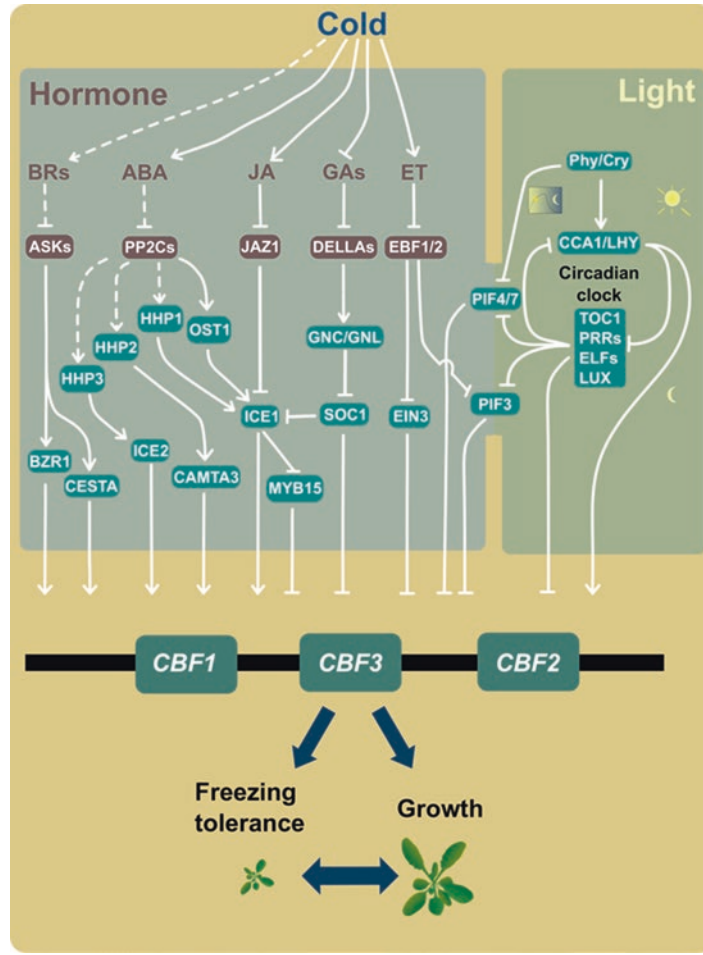

Fig. 1.2 The promoters of *CBF* genes constitute regulatory hubs that integrate internal and external signals. The output of these regulatory hubs is defined by the levels of each CBF protein, which will determine the degree of freezing tolerance and growth exhibited by a plant under cold conditions. Crosstalk between different hormone signaling pathways (e.g., DELLAs-JAZ1 interaction) has been omitted for clarity

inadequate levels of CBF proteins are highly detrimental for plant performance. By means of the complex network described in Fig. 1.2, a fine-tuning of *CBF* expression is achieved to increase freezing tolerance minimizing adverse effects for growth and development.

1.5 Additional Layers of Regulation of *CBF* Expression

The preceding section describes the complex transcriptional regulation of *CBFs* mediated by a collection of transcription factors that can bind to the *CBF* promoters. Nonetheless, other elements also play important roles in the transcriptional regulation of *CBF* expression (Fig. 1.3). For instance, it has been reported that RNA polymerase II binding to CBF promoters is mediated by the epigenetic silencing factor RNA-DIRECTED DNA METHYLATION4 (RDM4) (Chan et al. 2015). The molecular mechanism of this control, however, awaits further study. Moreover, transcriptional regulation of *CBF* genes also seems to be determined by histone posttranslational modifications. In this sense, the loss of function of FVE, a subunit of the polycomb repressor complex 2 responsible for H3K27 histone trimethylation (H3K27me3), results in increased *CBF* mRNA levels (Kim et al. 2004). Intriguingly, a genome-wide analysis of histone modifications in *Arabidopsis* revealed an enrichment of the repressive H3K27me3 mark in the *CBF* promoters at warm temperatures (Sequeira-Mendes et al. 2014). Additional

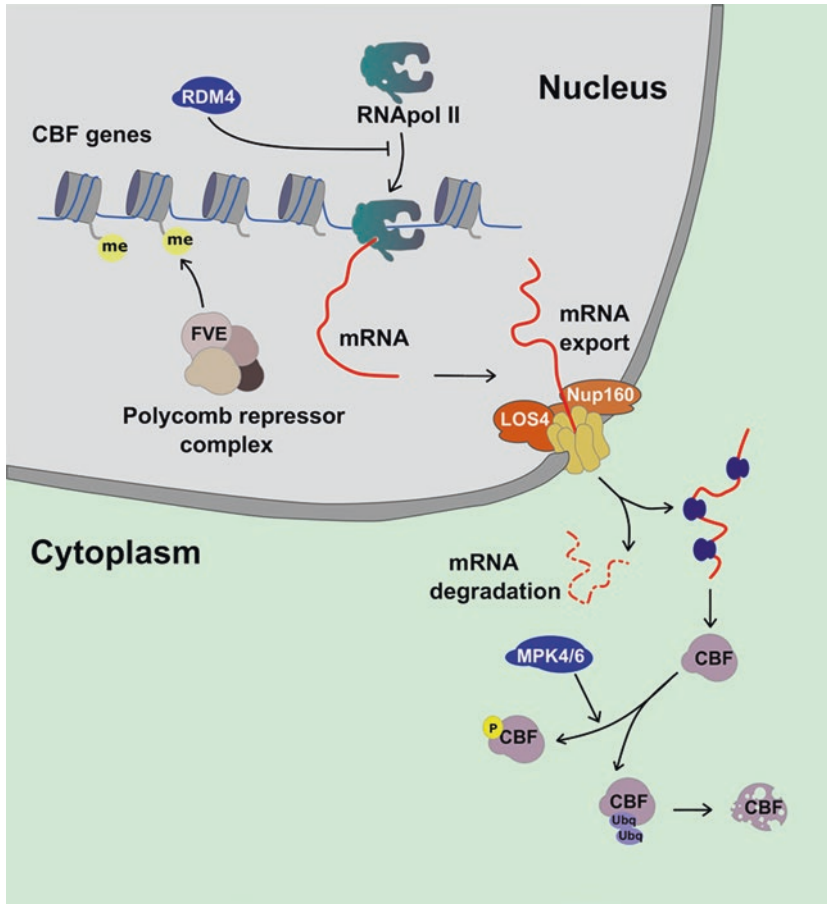

Fig. 1.3 Additional layers of regulation of *CBF* expression. Besides transcription factors, other elements play important roles in the regulation of *CBF* expression at transcriptional, posttranscriptional, and posttranslational level

research is needed to determine if this epigenetic landscape varies in response to low temperature and plays some role in regulating *CBF* transcription.

The expression of *CBFs* is also controlled downstream at posttranscriptional and posttranslational levels. Thus, several reports indicate that the coordination of mRNA export to the cytoplasm determines *CBF* expression. The lack of either LOW EXPRESSION OF OSMOTICALLY RESPONSIVE GENES4 (LOS4) or NUCLEOPORIN160 (NUP160), proteins that are located in the nuclear pore rim, results in aberrant nucleocytoplasmic mRNA partition, decreased *CBF* induction by low temperature, and reduced cold acclimation capacity (Gong et al. 2002, 2005; Dong et al. 2006). Furthermore, *CBF* expression might be also modulated through the control of mRNA stability. Although there is no evidence of such a regulation yet, it has been reported that *CBF* transcripts have a remarkably short half-life at warm temperatures (Zarka et al. 2003), which suggests there may be some factor(s) regulating their stability. In fact, recent studies have demonstrated that this is an important layer of gene expression regulation, specifically during cold acclimation (Perea-Resa et al. 2016). Hence, it would be worthwhile to study the stability of *CBF* transcripts.

On the other hand, multiple lines of evidence show that cold acclimation is regulated by posttranslational mechanisms (Barrero-Gil and Salinas 2013), and two posttranslational modifications affecting CBF proteins, namely, phos-

phorylation and ubiquitination, have been reported to date. A high-throughput phosphoproteomic analysis identified CBF1 and CBF3 as substrates of MITOGEN-ACTIVATED PROTEIN KINASE4 (MPK4) and MPK6 kinases in plants growing under control conditions (Popescu et al. 2009). The putative effect of this phosphorylation on CBF1 or CBF3 function, however, remains unknown. In a recent work, Liu and colleagues (Liu et al. 2017) have demonstrated that CBFs are subjected to degradation by the 26S proteasome and that 14-3-3 proteins positively regulate this process (Liu et al. 2017). From these results a working model has been proposed in which low temperature promotes 14-3-3 protein migration to the nucleus where they would interact with CBFs to stimulate their binding to an unknown E3 ubiquitin ligase, targeting them for degradation by the 26S proteasome pathway (Liu et al. 2017).

1.6 The Structure and Mode of Action of CBFs

CBF genes share significant sequence identity (approximately 85%) and are characterized by an APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) domain that connects them to the superfamily of AP2/ERF transcription factors, which is composed in *Arabidopsis* by 145 proteins. The AP2/ERF domain is, approximately, 60 amino acids long and originates a structure consisting of a three-stranded beta sheet parallel to an alpha helix that binds to GC-rich boxes in promoter sequences. Moreover, sequence analysis has identified short fragments flanking the AP2/ERF domain that are also required to bind to the CCGAC motif of the CRT/DRE sequence characteristic of the CBF targets. Other important domains within the CBF sequences include an aminoterminal nuclear localization signal and an acidic domain that is important for their transcriptional activation function (Stockinger et al. 1997). The study of the latter revealed another important feature concerning CBF function. Since acidic domains can interact with histone acetyltransferases, the ability of *Arabidopsis* CBF1 to interact with these chromatin modifiers was analyzed, revealing that some subunits of the SAGA histone acetyltransferase complex, including ALTERATION/DEFICIENCY IN ACTIVATION2A (ADA2A), ADA2B, and GENERAL CONTROL NONDEREPRESSIBLE5 (GCN5), can interact in vitro with this CBF (Stockinger et al. 2001). In addition, it was subsequently demonstrated that these proteins are required for inducing CBF-regulated gene expression (Vlachonasios et al. 2003). Indeed, histone acetylation status has repeatedly been shown to be important for cold-induced gene expression and the cold acclimation process. Whereas histone deacetylation at the cold-inducible promoters leads to gene repression (Kim et al. 2004; Jeon and Kim 2011), histone acetylation is associated with transcriptional activation (Pavangadkar et al. 2010). Thus, these results suggest that the function of CBFs as transcriptional activators may be mediated through the modification of chromatin structure, though the molecular details of this process need further research.

1.7 The Function of the CBFs in Cold Response

The role of CBFs in cold acclimation is to integrate a series of environmental cues and initiate, if appropriate, a transcriptional response that allows the plant to increase its freezing tolerance. As already mentioned, CBF proteins perform this function through their binding to a C-repeat motif, CCGAC, present in the promoter of many cold-induced genes, activating their transcription. Transcriptional activation, in turn, requires histone acetylation and depletion of nucleosomes (Venkatesh and Workman 2015). CBFs can interact with members of the SAGA complex to increase the acetylation levels of CBF-target genes around the C-repeat DNA-binding sites, a posttranslational modification that is necessary but not sufficient to activate their gene expression (Pavangadkar et al. 2010). It remains to be elucidated the role of CBF binding in nucleosome occupancy. After chromatin remodeling, RNA

polymerase II (RNApol II) needs to be recruited to activate transcription. Recent studies have characterized a multiprotein complex named mediator that plays a fundamental role in RNApol II recruitment to promoter sequences and activation of transcription in plants (Bäckström et al. 2007). It has been demonstrated that mediator subunits MED2, MED14, and MED16 are required to recruit RNApol II to CBF-responsive promoters (Hemsley et al. 2014). These particular mediator subunits bind to C-repeat-containing promoters in response to low temperature, and, although still is not known which factors determine this binding, CBFs are strong contenders.

Transcriptomic analysis of *Arabidopsis* triple *cbf* mutants has shown that loss of function of all CBF proteins affects the cold regulation of 447 genes (Jia et al. 2016; Zhao et al. 2016). Of these, 363 genes are upregulated, while 84 genes are downregulated (Zhao et al. 2016; Jia et al. 2016). Interestingly, 229 out of the 363 (63%) CBF-upregulated genes contain a CCGAC box in their promoter sequences. The remaining 134 genes either contain an unknown *cis*-element to which CBF proteins can bind or they represent CBF indirect targets. In contrast, only 21 out of the 84 (25%) CBF-downregulated genes contain the CCGAC *cis*-element. These data suggest that the primary role of CBF proteins during the cold response is to activate gene expression. The functional classification of the CBF-upregulated genes provides interesting insights into the processes regulated by this cold signaling pathway (Jia et al. 2016; Zhao et al. 2016). Indeed, 34 transcription factors are found in this list, revealing the amplificative nature of the CBF function in cold acclimation. Furthermore, 32 genes are related to carbohydrate metabolism, 24 to lipid metabolism, and 16 to cell wall modification (Jia et al. 2016; Zhao et al. 2016), which illustrates some of the metabolic and biochemical adjustments mediated by CBFs to increase freezing tolerance. In this sense, metabolic profiling of *Arabidopsis* plants has identified increased levels of sucrose and raffinose during this adaptive response (Kaplan et al. 2007), and several genes encoding enzymes involved in the biosynthesis of these sugars are among the set of CBF-inducible genes (Jia et al. 2016; Zhao et al. 2016). Consistent with these results, the overexpression of *CBFs* results in plants that accumulate these sugars even at warm temperatures (Gilmour et al. 2004). Similarly, it is well established that cold exposure induces proline accumulation in *Arabidopsis* (Kaplan et al. 2007) and a gene that encodes one of the key enzymes for the synthesis of this amino acid *DELTA-1-PYRROLINE-5-CARBOXYLATE SYNTHASE2* (*P5CS2*) is regulated by the CBF transcription factors (Zhao et al. 2016; Jia et al. 2016). Again, the overexpression of *CBF* genes results in plants that accumulate proline even at warm temperatures (Gilmour et al. 2004). Cold acclimation is also associated with changes in lipid membrane composition (Uemura et al. 1995) to prevent damage induced by freezing temperatures (Ruelland et al. 2009). It is likely that some of the CBF-inducible genes related to lipid metabolism are involved in this process. Another well-documented response to cold in plants is the accumulation of hydrophilic proteins (late embryogenesis abundant proteins and dehydrins) (Ruelland et al. 2009). The *Arabidopsis* CBF transcription factors are responsible for the cold induction of some of these proteins, including COLD INDUCIBLE1 (KIN1), KIN2, LATE EMBRYOGENESIS ABUNDANT4-5 (LEA4-5), LOW TEMPERATURE-INDUCED30 (LTI30), COLD REGULATED47 (COR47), and EARLY RESPONSIVE TO DEHYDRATION10 (ERD10) (Jia et al. 2016; Zhao et al. 2016).

As discussed above, *CBF* expression is tightly regulated. *CBF* transcripts accumulate quickly after cold exposure, eliciting a transcriptional cascade that results in increased freezing tolerance. However, the cold induction of *CBFs* is transient, and the levels of the corresponding mRNAs and proteins return to resting levels after a few hours into the cold response (Novillo et al. 2007; Liu et al. 2017). It has been proposed that the cold induction of *CBFs* needs to be transient since constitutive high levels of *CBF* transcripts lead to growth retardation, dwarfism, and delayed flowering in plants growing at warm temperature (Liu et al. 1998; Gilmour et al. 2004). A molecular mechanism for this phenotype was outlined

when it was discovered that constitutive overexpression of *CBF1* led to accumulation of DELLA proteins (Achard et al. 2008), which are well known for restraining plant growth (Harberd et al. 2009). In this context, it was shown that overexpression of *CBF1* increases the expression of two genes, *GIBBERELLIN 2-OXIDASE3* (*GA2ox3*) and *GA2ox6*, encoding enzymes that deactivate gibberellins, causing the accumulation of DELLAs (Achard et al. 2008). From these results, it was proposed that one of the functions of CBFs was to restrain plant growth through negative regulation of gibberellin synthesis, which would contribute to increase survival to freezing temperatures (Achard et al. 2008). Intriguingly, neither *GA2ox3* nor *GA2ox6* is affected in mutant plants lacking CBF function, and, more importantly, these mutants also show retarded growth (Jia et al. 2016; Zhao et al. 2016). These observations suggest that CBFs should also play a role in plant development.

Finally, an important question regarding the function of CBFs is whether or not all of them play the same role in the development of freezing tolerance during cold acclimation. Based on a number of observations, it has been proposed that CBF function is largely redundant. First, similar sets of genes are upregulated in transgenic lines overexpressing different *CBF* genes (Gilmour et al. 2004) as well as in single CRISPR *cbf* mutants (Shi et al. 2017). Second, transgenic lines overexpressing different *CBF* genes show a similar profile of metabolites with high accumulation of proline and sugars (Gilmour et al. 2004). Third, the double *cbf1cbf3* mutant but not the single mutants *cbf1* and *cbf3* shows an altered capacity for cold acclimation (Zhao et al. 2016; Jia et al. 2016). Fourth, many of the *CBF* regulators described so far can bind to any of the *CBF* promoters, indistinctively in electrophoretic mobility shift assay (EMSA) or chromatin immunoprecipitation (CHIP) assays, which suggest a similar regulation for the *CBF* genes (Table 1.1). Other findings, however, suggest that there may be some divergence in the function of CBFs. Thus, (1) some experiments have revealed subtle differences in expression patterns between *CBF1* and *CBF3* on one side and *CBF2* on the other side, with the latter showing a slower cold upregulation and wider tissue expression pattern (Novillo et al. 2007); (2) while CRISPR *cbf1* and *cbf3* single mutants display WT-like capacity to cold acclimate, the capacity of the CRISPR *cbf2* single mutant is significantly reduced compared to WT plants (Zhao et al. 2016); (3) as already mentioned, CBF1 and CBF3, but not CBF2, are targets of MAP kinases and, furthermore, are subjected to 14-3-3 protein regulation (Popescu et al. 2009; Liu et al. 2017); and (4) a survey of natural variation for freezing tolerance in 477 European populations of *Arabidopsis* found frameshifts and premature stop codon mutations associated with adaptation to warmer climates in the sequences of *CBF2* and *CBF3* genes, but remarkably not in that of *CBF1* (Monroe et al. 2016). Indeed, some authors have suggested that CBF2 may have a different function than CBF1 and CBF3 (Novillo et al. 2004; Zhao et al. 2016).

Despite the intense research carried out during the last decades, the particular function of each CBF and the interactions among them are not clear yet because there are conflicting results regarding the phenotype of mutant lines. While a T-DNA inserted in the promoter of *CBF2* causes upregulation of *CBF1* and *CBF3* genes, and an enhancement of cold acclimation capacity (Novillo et al. 2004), frameshift mutations created using CRISPR technology in the *CBF2* sequence cause a reduction of cold acclimation capacity without significant impact on the expression of *CBF1* or *CBF3* (Zhao et al. 2016). A similar situation has been observed when analyzing the effect of *cbf1cbf3* double mutations. It has been reported that a large deletion encompassing *CBF1* and *CBF3 loci* leads to an increase of *CBF2* expression in response to low temperature and a concomitant increase in freezing tolerance (Zhao et al. 2016). In contrast, a frameshift mutation of *CBF1* combined with a T-DNA insertion in the *CBF3* coding sequence did not increase *CBF2* expression after cold exposure and resulted in a reduced ability to cold acclimate (Jia et al. 2016). These conflicting results reveal that the molecular nature of *CBF* mutant

alleles has a definitive influence on the regulation of *CBF* genes. Additional studies are needed to elucidate the complex relationships between the *CBF* genes.

1.8 The Role of CBFs in Plant Response to Cold-Related Abiotic Stresses

Heretofore we have discussed the role of CBF proteins in the context of cold acclimation. Indeed, since their original identification, the *CBF* genes were characterized as specifically induced by cold but not by other cold-related abiotic stresses like drought, salt, or high osmotic conditions (Gilmour et al. 1998; Liu et al. 1998; Medina et al. 1999). However, early studies also demonstrated that overexpression of *CBFs* increases tolerance to salt and drought stresses in *Arabidopsis* (Liu et al. 1998; Kasuga et al. 1999), which suggested that CBFs might also have a role in other abiotic stress responses apart from cold acclimation. Additional experiments provided support to this idea by demonstrating that ABA induces *CBF* expression in certain conditions (Knight et al. 2004) since it is well known that ABA increases in response to different abiotic stresses in addition to low temperature, including drought, salt, and high osmotic conditions (Walton 1980; Lang et al. 1994; Perea-Resa et al. 2016). Genetic evidence on the implication of CBFs in salt and drought tolerance was supplied by the phenotypic analysis of the effects of a T-DNA insertion in the *CBF2* promoter which revealed that, besides increasing *CBF1* and *CBF3* expression as well as freezing tolerance, this mutation also boosted salt and dehydration tolerance (Novillo et al. 2004). Lastly, recent reports have shown that loss of function of all three CBFs in *Arabidopsis* results in decreased salt tolerance (Zhao et al. 2016). These results firmly establish that CBFs play a role in other abiotic stress responses beside cold acclimation, which, after all, is not surprising considering that plant responses to cold, salt, and water stresses are quite related (Chinnusamy et al. 2004) and several works have found a significant crosstalk among these responses (Nakashima et al. 2014). Nonetheless, the precise contribution of CBFs to these stress responses and the specific mechanisms controlled by these transcription factors remain to be determined.

1.9 The Role of CBFs in Plants That Do Not Cold Acclimate

We have mentioned earlier that *CBF* genes have been identified in a wide range of plants, including some species that are freezing-sensitive and cannot develop a cold acclimation response, such as rice (Xue 2003), tomato (Zhang et al. 2004), and maize (Qin et al. 2004). These findings raise the question of what could be the function of CBFs in these organisms. An interesting hypothesis already discussed is that CBFs may have additional roles in plant development (Zhao et al. 2016). Thus, it might be possible that the major role of CBFs in freezing-sensitive species is restricted to their involvement in development. Alternatively, the function of CBFs in freezing-sensitive species might be connected with a role in salt or drought responses.

It is worth noting, however, that a limited but significant capacity to increase constitutive tolerance to chilling temperatures after exposure to suboptimal growth temperatures has been reported in freezing-sensitive species such as maize (Nie et al. 1992), rice (Kuk et al. 2003), and tomato (Barrero-Gil et al. 2016). This response, which has been termed chilling acclimation (Anderson et al. 1994), has been the subject of a recent study in tomato which shows that it shares many characteristics with cold acclimation, including a large transcriptomic rearrangement (Barrero-Gil et al. 2016). Therefore, it is tempting to speculate that CBF proteins in freezing-sensitive species may be responsible for the elicitation of this transcriptomic response in a similar way as they regulate cold acclimation in freezing-tolerant species. Indeed, tomato *CBF* expression is induced by suboptimal growth temperatures (Barrero-Gil et al. 2016). Furthermore, ectopic expression of tomato *SlCBF1* increases freezing tolerance in transgenic *Arabidopsis*

(Zhang et al. 2004), and overexpression of *Arabidopsis AtCBF1* increases constitutive chilling tolerance in tomato (Hsieh et al. 2002) and rice (Oh et al. 2005). These findings indicate that tomato and *Arabidopsis* CBF1 homologs have some common functional characteristics. A recent study has shown that SlCBF1 is a positive regulator of tomato constitutive chilling tolerance (Wang et al. 2015). Thus, it is reasonable to expect that CBFs may have a role in the development of chilling acclimation in tomato and possibly in other freezing-sensitive species.

1.10 Conclusions and Perspectives

The past two decades have witnessed intense research focused on unveiling the molecular mechanism underpinning the cold acclimation response. These efforts have led to the discovery and description of a signaling pathway centered on the function of a small family of transcription factors called CBFs. Natural variation and genetic studies show that these transcription factors are indeed critical for full development of that response. The regulation of *CBF* expression is extremely complex and tightly regulated, positively or negatively, by multiple intermediates and mechanisms operating at many different levels. Our current state of knowledge establishes that CBFs regulate one of the most important signaling pathways controlling cold acclimation.

In spite of all these findings, there is a great deal of unknown aspects concerning CBF function. For instance, the regulation of *CBF* expression integrates multiple signaling pathways from internal and external cues, but how is this integration carried out and how does it relate to the output of this signaling pathway? Which is the precise role of CBF proteins in plant growth and development? Which are the mechanisms controlled by CBF proteins in abiotic stress responses different from cold acclimation? Which are the specific contributions of each CBF protein to plant development and abiotic stress responses? These questions are of utmost relevance not only for a basic understanding of plant biology but also from an applied perspective. Although more studies are necessary to fully harness CBF biotechnological potential, several field studies have already provided promising data showing that inducible overexpression of *CBF* genes can be a successful strategy to increase crop tolerance to freezing temperatures (Artlip et al. 2014) and drought (Xiao et al. 2009; Datta et al. 2012; Bhatnagar-Mathur et al. 2014). Successful transfer of knowledge from the lab to the field is proving to be slower than what was estimated 20 years ago (Thomashow 2010; Varshney et al. 2011). One of the reasons for this delay is the complexity of abiotic stress responses for which, no doubt, the CBF signaling pathway can serve as a paradigm (Thomashow 2010). However, the degree of knowledge achieved in the past decade combined with new DNA editing techniques should soon allow for a rational design of crops with enhanced tolerance to abiotic stress. In this scenario, we believe that CBF research is going to play a very important role.

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RNA Regulation in Plant Cold Stress Response

2

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Abstract

In addition to plants, all organisms react to environmental stimuli via the perception of signals and subsequently respond through alterations of gene expression. However, genes/mRNAs are usually not the functional unit themselves, and instead, resultant protein products with individual functions result in various acquired phenotypes. In order to fully characterize the adaptive responses of plants to environmental stimuli, it is essential to determine the level of proteins, in addition to the regulation of mRNA expression. This regulatory step, which is referred to as "mRNA posttranscriptional regulation," occurs subsequent to mRNA transcription and prior to translation. Although these RNA regulatory mechanisms have been well-studied in many organisms, including plants, it is not fully understood how plants respond to environmental stimuli, such as cold stress, via these RNA regulations.

A recent study described several RNA regulatory factors in relation to environmental stress responses, including plant cold stress tolerance. In this chapter, the functions of RNA regulatory factors and comprehensive analyses related to the RNA regulations involved in cold stress response are summarized, such as mRNA maturation, including capping, splicing, polyadenylation of mRNA, and the quality control system of mRNA; mRNA degradation, including the decapping step; and mRNA stabilization. In addition, the putative roles of messenger ribonucleoprotein (mRNP) granules, such as processing bodies (PBs) and stress granules (SGs), which are cytoplasmic particles, are described in relation to RNA regulations under stress conditions. These RNA regulatory systems are important for adjusting or fine-tuning and determining the final levels of mRNAs and proteins in order to adapt or respond to environmental stresses. Collectively, these new areas of study revealed that plants possess precise novel

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regulatory mechanisms which specifically function in the response to cold stress.

Keywords

RNA regulation · Posttranscriptional regulation · mRNA degradation · mRNA stabilization · Cold stress response

Abbreviations

CA	Cold acclimation
CBF	C-repeat-binding factors
DA	Cold de-acclimation
DREB	Dehydration-responsive element-binding proteins
hnRNPs	Heterogeneous nuclear ribonucleoproteins
mRNP	Messenger ribonucleoprotein
NMD	Nonsense-mediated decay
PBs	Processing bodies
PTC	Premature termination codon
RBPs	RNA-binding proteins
RRM	RNA recognition motif
SGs	Stress granules
snRNP	Small nuclear ribonucleoprotein particle
SR	Serine-/arginine-rich

2.1 Introduction

Environmental stresses, such as temperature changes, dehydration, and high salinity conditions, affect the distribution, growth, and productivity of not only land plants but also many organisms including animals. Low, chilling, or freezing temperatures are some of the most prevalent environmental factors which directly affect enzyme activity and therefore impact adaptation or tolerance mechanisms that are essential for survival. This is especially important in plants since they are incapable of moving from harmful environmental conditions.

In temperate zones, many plants are capable of perceiving changes in the seasons through variations in temperature and other conditions such as day length. In many plants, the perception and response to these cues are directly involved in the determination of flowering timing and fruiting. In addition, the perception of temperature downshifts also enables plants to trigger adaptations which increase tolerance to low-temperature stress. This is especially true for overwintering plants that possess an ability to adapt to chilling or freezing temperatures via a process termed as cold acclimation.

During autumn, when plants are exposed to periods of nonfreezing low temperatures, the cold acclimation (CA) trait is acquired which enables overwintering plants to obtain freezing tolerance. In herbaceous plants, periods of cold acclimation and growth retardation are often closely related during periods of overwintering. During the subsequent spring months, plants resume growth after plants sense temperature upshifts during a process which is termed cold de-acclimation (DA). In contrast to the process of CA, the acquired levels of freezing tolerance are rapidly diminished during the DA process (Fowler and Thomashow 2002).

In studies which aimed to decipher the mechanisms of CA and DA at the gene and protein level, many cold-inducible genes and gene products, including the dehydration-responsive element-binding proteins/C-repeat (CRT)-binding factors (DREB/CBF) regulon, were analyzed and functionally characterized (Maruyama et al. 2004). During CA, cold-induced transcripts are translated into proteins with specific functions for increasing freezing tolerance or adaptation to low-temperature stress. Recently, comprehensive transcriptomic analyses using microarray and/or next-generation sequencing technologies have generated large datasets which have enabled scientists to acquire a much more comprehensive understanding of the mechanisms and phenomena associated with the CA and DA processes in plants (Maruyama et al. 2004; Lei et al. 2014; Zhao et al. 2016). In addition to the genome-wide studies at the transcript level, extensive proteomic analyses using two-dimensional protein gel analyses or shotgun proteomics approaches by mass spectrometry have greatly improved our understanding of these pro-

cesses (Bae et al. 2003; Cui et al. 2005; Nakaminami et al. 2014; Shi et al. 2014). In the post-genomic era, these high-throughput analyses of the transcriptome and proteome are important to enable the identification of key steps or pathways of plants. However, the resultant comprehensive datasets that are generated from these two approaches are not completely concordant with one another (Nakaminami et al. 2014). Additionally, it is also known that gene and protein expression patterns likely differ from one another due to the posttranscriptional or translational regulation which occurs after transcription and before translation. Collectively, these mechanisms are very important regulatory steps which directly affect the level of mRNAs and proteins (Floris et al. 2009).

In order to generate a wide range of responses which enable plants to quickly adapt to their dynamic and variable environmental conditions, precise regulatory mechanisms are very important to enable the fine-tuning of the expression of genes and proteins. Posttranscriptional RNA regulation is a highly coordinated mechanism that functions to directly affect and regulate the cellular levels of mRNAs and proteins (Yeap et al. 2014). Subsequent to the transcription of genes, mRNA maturation steps such as splicing, capping, and adenylation are important for the stabilization of mRNAs. In order to assess the quality of mRNA, ribosomal RNA and other RNA-binding proteins scan mRNA to avoid translating aberrant mRNAs. In the event that errors are present in mRNA molecules, the mRNAs are rapidly degraded. In addition to this degradation process, mRNAs, which are supposed to be eliminated, are also degraded for adjusting the appropriate amount of mRNA/protein levels. These degradation regulations are important since the level of mRNAs is determined by the balance of the speed of RNA transcription and degradation (Perez-Ortin et al. 2007). mRNA stabilization is also an important mechanism which affects the levels of mRNA and translation. Stabilized mRNAs are protected from degradation by specific RNA-binding proteins. Taken together, mRNAs are regulated by several steps after the point of transcription prior to the initiation of translation. In plants, gene and protein expression are regulated by these posttranscriptional activities, which ultimately affects the ability of plants to adapt to environmental changes or changes of the seasons. Here, we summarize these RNA regulations, such as degradation or stabilization mechanisms, to shed light on their functional involvement in cold stress responses.

2.2 Posttranscriptional Regulation

During gene expression in response to specific environmental changes or stimuli, RNAs are transcribed from genomic DNA in the nucleus, and primary transcribed RNAs are subsequently converted into mature mRNAs (Fig. 2.1). This primary transcribed RNA molecule is called "pre-mRNA," which is a precursor of mRNA. The maturation steps of pre-mRNA into mature mRNA molecules are referred to as "RNA processing." In general, the RNA processing steps are initiated with capping and are followed by splicing and/or polyadenylation steps (Yeap et al. 2014).

The first step of RNA processing is the addition of a 7-methylguanosine cap (m7G-cap) to the 5′ end of pre-mRNA in a process referred to as "capping." The function of the cap is to prevent the degradation of the mRNA molecule. In addition, this cap structure is a marker for mRNA export outside of the nucleus or for the initiation of translation. The other processing step is splicing, which removes introns from pre-mRNA. In eukaryotes, introns are commonly present in the majority of genes, and they are characterized as nonprotein coding sequences which need to be removed for the generation of mature mRNA molecules. Polyadenylation is also an additional RNA processing step which involves the addition of a poly(A) sequence to the 3′ end of mRNA. After RNA processing, matured mRNAs are transported from the nucleus to the cytosol, and the quality of mRNAs is checked prior to the initiation of translation (Fig. 2.2). This step in the process is known as the quality control system of mRNA, which is a surveillance mechanism that

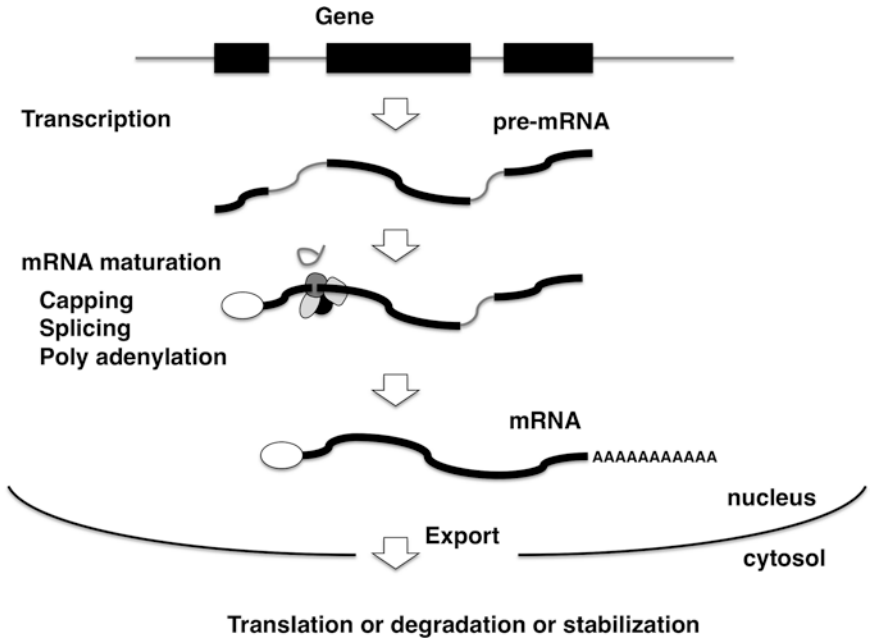

Fig. 2.1 Schematic illustration of transcription through the mRNA maturation step. After transcription, pre-mRNAs are matured by capping, splicing, and polyadenylation in the nucleus

eliminates inappropriate RNAs in order to prevent the translation of mRNAs into nonfunctional proteins from dysfunctional RNA sources. In this quality control step, aberrant mRNAs are rapidly degraded, and normal mRNAs are translated into proteins. It is important to note that not all transcribed mRNAs are translated into proteins, especially under stress conditions or during periods of response to environmental changes. Plants have developed conservative processes which prevent the translation of unnecessary proteins and the protection of mRNA molecules until they are needed. In plant cells, nontranslating mRNAs are found within two types of messenger ribonucleoprotein (mRNP) granules in cytosol. The first is referred to as processing bodies (PBs), which are subcellular structures where aberrant mRNAs or mRNAs, which are supposed to be eliminated, are degraded by cellular machinery such as decapping enzymes and nucleases (Fig. 2.2) (Bailey-Serres et al. 2009; Xu and Chua 2011). PBs are present under both normal and stress conditions. The second type of mRNP granule is known as stress granules (SGs), which are cytoplasmic particles containing mRNAs and RNA-binding proteins, including translation initiation components which function to protect mRNAs until translation (Fig. 2.2). In contrast to processing bodies, SGs appear specifically under stress conditions and are co-localized with PBs (Buchan et al. 2008; Weber et al. 2008; Anderson and Kedersha 2009).

During transcription and posttranscriptional processes, mRNAs are always associated with RNA-binding proteins (RBPs) (Lorkovic 2009; Ambrosone et al. 2012), which are key regulators of these processes in plants. These RNA-binding proteins are referred to as heterogeneous nuclear ribonucleoproteins (hnRNPs), and they interact with pre-mRNA or mRNA within mRNP complexes. In plants, there are hundreds of functional RNA-binding proteins; however the precise roles for all RNA-binding proteins during posttranscriptional regulation under stress conditions still remain to be clearly elucidated.

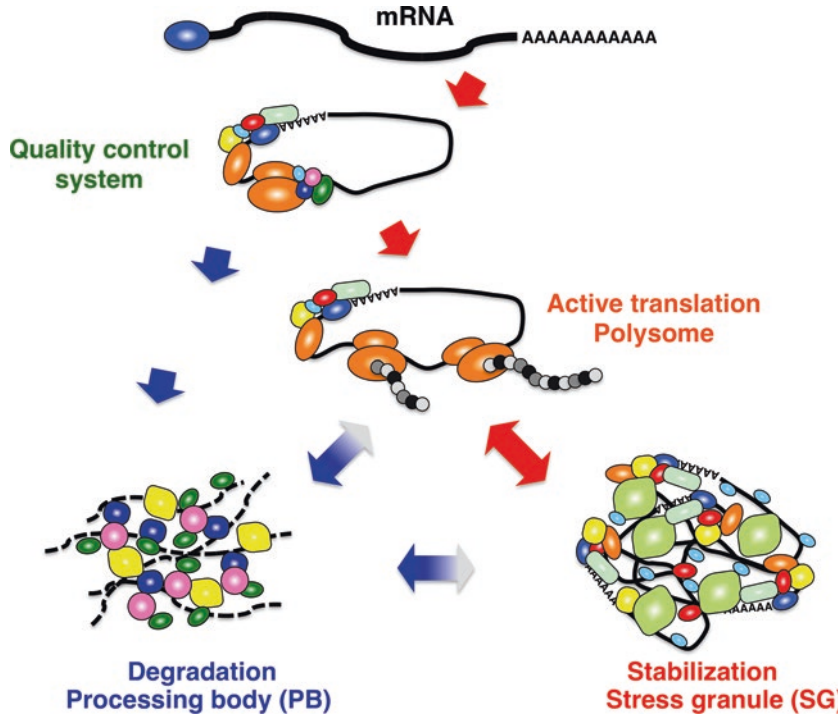

Fig. 2.2 Schematic illustration of mRNA quality control to translation and degradation or stabilization. Mature mRNAs are checked by the surveillance mechanism to eliminate aberrant mRNA by nonsense-mediated decay (NMD). The correct form of mRNA is translated into protein by an active translational complex, polysome. Under stress conditions, processing bodies (PBs) and stress granules (SGs) are formed in the cytosol. PBs are involved in mRNA degradation, and SGs function to protect or stabilize mRNA. Aberrant mRNAs found by the NMD pathway are also degraded in PB

2.3 mRNA Maturation

In eukaryotes, gene transcription includes several co-transcriptional processes such as mRNA capping, splicing, and polyadenylation as previously described. In response to cold stress in plants, this mRNA processing is the first step to regulate mRNA levels after transcription. The alternative splicing step has been well-studied and characterized the functions of splicing factors during cold stress.

2.3.1 Capping and Polyadenylation

Capping is an initiation step of mRNA processing, which involves the cleavage of the 5′ triphosphate of the pre-mRNA and the addition of 7-methylguanosine (m7G-cap) at the 5′ -end of mRNA (Shuman 2015). This step usually occurs simultaneously with transcription and is also called co-transcriptional capping.

It has been reported that SHINY1 and 4 (SHI1, SHI4) are involved in RNA capping and the regulation of gene expression during exposure to cold and other stresses (Jiang et al. 2013). The SHI1 protein is a RNA-binding protein containing a K homology domain which interacts with SHI4 (also known as FIERY2 (FRY2)/C-terminal domain phosphatase-like1 (CPL1)) in the nucleus. SHI4/FRY2/CPL1 was characterized as a C-terminal domain (CTD) phosphatase and interacts with the CTD of RNA polymerase II (Koiwa et al. 2004; Hausmann et al. 2005). Phosphorylation and dephosphorylation of the CTD of RNA polymerase II regulate co-transcriptional mRNA capping. As reported by Jiang et al. (2013), both *shi1* and *shi4* mutant plants exhibited chilling-sensitive phenotypes.

SHI1 and 4 negatively regulated the capping of cold-responsive genes such as *cold-regulated* (*COR*) *15A* and *COR47* and altered the polyadenylation site selection for some of the stress-inducible genes, resulting in an alteration of gene expression. The constitutively expressed *SHI1* and *4* genes both function in the regulation of the capping of genes in relation to cold stress, and their expression patterns are not changed in response to cold stress treatment. In the *shi1* and *shi4* mutants, the capping step of cold-responsive genes was misregulated, resulting in a cold-sensitive phenotype. These data provided a clear indication that co-transcriptional capping is important for gene expression and stress responses.

A previous study has shown that a polyadenylation factor responds to other stresses (not cold stress) and alters the polyadenylation site of mRNA (Zhang et al. 2008). Although it is possible that polyadenylation might be regulated by cold stress, few reports have described a relationship of polyadenylation and the cold stress response. Overall, the role of mRNA capping and the regulation of polyadenylation in the cold stress response still remain largely unclear at this time.

2.3.2 Splicing and Alternative Splicing

2.3.2.1 Overview of Splicing and Alternative Splicing

After the addition of a cap structure and/or polyadenylation, introns are subsequently removed in a process called pre-mRNA splicing in order to produce mature mRNAs. In most cases, eukaryotic pre-mRNAs contain introns, which are non-protein coding sequences, and pre-mRNA splicing is a crucially important mechanism that is necessary to produce functional proteins. In the case of mammals or budding yeast, not all splice sites within a pre-mRNA are used for the production of mature mRNA. In order to greatly increase the functional diversity of proteins that are produced, multiple variations of mRNAs are capable of being synthesized from single genes or the same pre-mRNA. This process of conditional splicing is termed alternative splicing, which is characterized by various splicing events which ultimately lead to the generation of diverse mature transcripts (Smith and Valcarcel 2000; Blencowe 2006; Kim et al. 2007a; Nilsen and Graveley 2010; Witten and Ule 2011).

In eukaryotes such as plants, mammals, and budding yeast, different types of alternative splicing events, such as exon skipping, alternative 5′ or 3′ splice site selection, and intron retentions, have diverse effects on proteins, ultimately resulting in significant changes or the deletion of domains in functional proteins. As a consequence of these different types of splicing or a combination of these events, an enormous amount of protein diversity can be created to provide a range of functionality related to developmental processes or the response to environmental conditions. In addition, it is possible that these different splicing events might result in aberrant mRNA production which contains a premature termination codons (PTCs). In these cases, mRNAs that contain PTCs are capable of being translated into truncated proteins, or the aberrant mRNAs may also be degraded by the nonsense-mediated decay (NMD) pathway (Filichkin et al. 2010; Nicholson et al. 2010; Leviatan et al. 2013) (please refer to 2.3.3). Thus, alternative splicing, including alternative NMD, regulates the level of the correct form of mRNAs, resulting in regulation of the level of functional proteins.

In plants, alternative splicing occurs in developmental processes and stress responses; however, detailed knowledge pertaining to alternative splicing events is still limited. Several protein kinases, transcription, and splicing factors have been reported to be involved in alternative splicing in response to abiotic stresses (Mastrangelo et al. 2012). In *Arabidopsis*, data generated from high-throughput sequencing analyses have shown that 61% of multiexonic genes are alternatively spliced under normal conditions (Marquez et al. 2012). With respect to temperature stress exposure, both cold and heat stress result in significant increases of alternative splicing of many genes (Lazar and Goodman 2000; Iida et al. 2004;

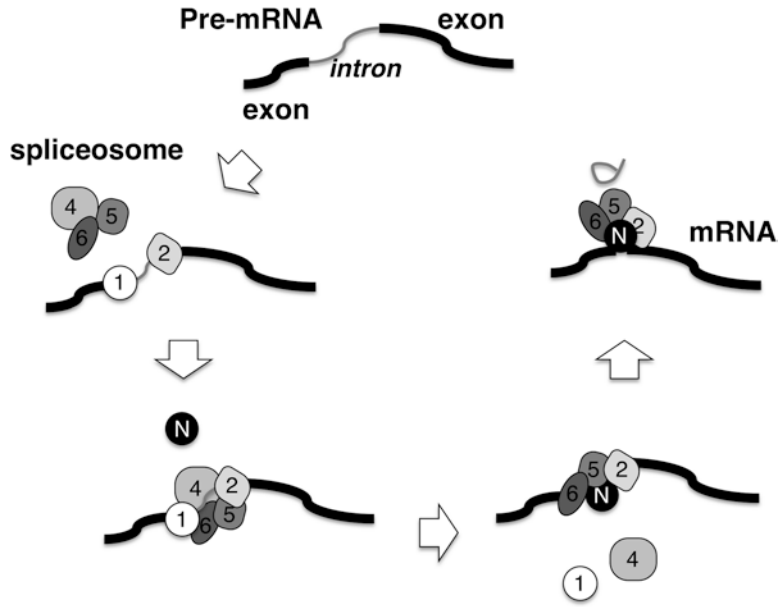

Fig. 2.3 Schematic illustration of the basic splicing pathway by spliceosomes. The U1 snRNP complex recognizes the 5′-splice site, and U2 snRNP binds to the 3′-splice site. U4/U6.U5 snRNP bound to U2 complex followed by NTC complex binding. The U1 and U4 complexes are released and the intron sequence is spliced out. 1, U1 complex; 2, U2 complex; 4, U4 complex; 5, U5 complex; 6, U6 complex; N, NineTeen-Related Complex (NTR)

Palusa et al. 2007; Filichkin et al. 2010; Leviatan et al. 2013; Reddy et al. 2013). Thus, alternative splicing has been recognized as an important step of RNA regulation in relation to the cold stress response of plants.

Pre-mRNA splicing is mediated by a ribonucleoprotein complex, which is known as a spliceosome (Fig. 2.3) (Kornblihtt et al. 2013). Spliceosomes are composed of U-rich small nuclear RNAs (snRNAs; U1, 2, 4, 5, and 6) and Sm ribonucleoproteins. The initiation of splicing is the recognition of a 5′-splice site by U1 small nuclear ribonucleoprotein particle (snRNP). In the next step, U2 snRNPs bind to the 3′-splice site and U4/U6. Subsequently, the U5 snRNP is then docked onto the U2 snRNP. After rearrangement of the spliceosome complex, U1 and U4 snRNPs are released, and NineTeen complex (NTC) or NineTeen-Related Complex (NTR), which is a non-snRNP, is recruited (Hogg et al. 2010). DEAD- or DEAH-type RNA helicases are involved in the rearrangement of the complex by reconfiguring RNA-RNA interactions (Cordin et al. 2012; Cordin and Beggs 2013; Meyer et al. 2015; Yan et al. 2015). NTC is essential for activation of the spliceosome, and NTR is involved in the disassembly of the spliceosome (Hogg et al. 2010; Chen et al. 2013a; Meyer et al. 2015; Fourmann et al. 2017). Lastly, the intron is cleaved and exons are ligated to produce mature mRNA.

In *Arabidopsis*, 395 genes/proteins related to the splicing process have been identified in silico, and 430 spliceosomal factors were identified and re-annotated based upon characterization by mass spectrometry (Wang and Brendel 2004; Koncz et al. 2012). The serine-/arginine-rich (SR) protein family, which is one of the regulators of splicing processes, is also involved in the binding specificity of spliceosome to pre-mRNAs. SR proteins consist of the RNA recognition motif (RRM), which is a RNA-binding motif, and a serine-/arginine-rich domain which is involved in protein-protein interaction (Palusa et al. 2007; Barta et al. 2010; Reddy et al. 2013). These spliceosomal or interaction proteins function in the regulation of splicing processes or alternative splicing in order to adapt to environmental conditions, such as cold stress.

2.3.2.2 Splicing Factors Related to Cold Stress Response

Stabilized1 (STA1), which is a U5-snRNP interacting protein, is induced in the cold stress response and is functionally involved in pre-mRNA splicing. *COR15A*, a DREB/CBF regulon

gene, is one target of STA1 (Lee et al. 2006; Kim et al. 2017), which functions to control the pre-mRNA splicing of *COR15A*. In the *sta1* mutant, *COR15A* pre-mRNA accumulates in response to cold stress. This mis-spliced *COR15A* contains a PTC and results in chilling and freezing sensitivity of *sta1* mutant plants. These data provide clear evidence that the splicing process of *COR15A* by STA1 is important for cold stress responses in plants (Lee et al. 2006; Guan et al. 2013).

Indeterminate domain 14 (IDD14), which is a transcription factor involved in starch metabolism, is another target of STA1 (Seo et al. 2011; Kim et al. 2017). Under cold conditions, an alternative spliced form of *IDD14*, *IDD14β*, which lacks a DNA-binding domain, increases, and heterodimers of *IDD14α* (full-size mRNA)-*IDD14β* form. Due to a reduction of gene expression of *Qua-Quine Starch* (*QQS*), which mediates starch degradation, these collective alterations result in increased amounts of starch in plants (Li et al. 2009), which is important for augmentations of soluble sugar and freezing tolerance of plants (Yano et al. 2005).

Pre-mRNA-splicing factors 31 (PRP31) is also another splicing factor that is associated with STA1, which functions to regulate the formation of the U4/U6.U5 complex (Du et al. 2015). PRP31 contains a Nop domain and c-terminal domain, which function in the selectivity of RNA and protein binding. In addition, they are also important for the assembly of the U4/U6.U5 spliceosome. Target candidates of PRP31 are cold-responsive genes, such as *responsive to desiccation 29 A* (*RD29A*), *COR6.6*, and *COR15A*. In the cold-sensitive *prp31* mutant, intron retention of these genes increases during cold stress treatment, indicating that mis-splicing occurred and is likely related to the reduced cold tolerance of the mutant. Similar to STA1, PRP31 is also important for the cold response in plants through its role in the splicing process.

Regulator of CBF gene expression1 (RCF1) is a cold-inducible DEAD-box RNA helicase, which functions to regulate the splicing process in the nucleus (Guan et al. 2013). The *rcf1* mutant is sensitive to both chilling and freezing stress and is characterized by the accumulation of mis-spliced forms of cold-inducible gene targets of RCF1, MYB family transcription factor *circadian1* (*CIR1*), *SPFH/PHB domain-containing membrane-associated protein* (*SPFH*), *pseudo-response regulator5* (*PRR5*), and *shaggy-like serine/threonine kinase12* (*SK12*). This misregulation is significant since CIR1 and SPFH are known positive regulators of cold stress response in plants, whereas PRR5 and SK12 are negative regulators. When *RCF1* was mutated, both positive and negative regulators for cold stress responses were affected. In addition, plants overexpressing *RCF1* exhibited enhanced chilling and freezing tolerance. At the present time, evidence suggests that the phenotypes of these *rcf1* and *RCF1*-ox plants are caused by the splicing or mis-splicing of positive regulators, such as *CIR1* or *SPHF* genes, and the splicing regulation of *PRR5* and *SK12* genes and the increased expression of DREB/CBF genes and DREB/CBF regulons are compensatory responses.

Similar to STA1 and RCF1, a U5 small nuclear ribonucleoprotein helicase, Bad response to refrigeration 2b (Brr2b), is another important protein for the splicing process (Guan et al. 2013). Brr2b is also cold-inducible and its mutant exhibited a chilling- and freezing-sensitive phenotype. Even if Brr2b is co-expressed with STA1 and RCF1, the splicing targets of these proteins are different, providing direct evidence that they function in different spliceosome components and pathways.

Arabidopsis glycine-rich RNA-binding proteins (AtGRP) 7 and 8 are cold-inducible RNA-binding domain proteins, which contain RRM-type RNA-binding domain and glycine-rich regions, and are known to function in the 5′ splice site selection. In *AtGRP7*-ox plants, target pre-mRNA contained an alternative 5′ splice site which resulted in the production of PTC. AtGRP8 also negatively regulates targets similar to AtGRP7 (Schoning et al. 2008; Streitner et al. 2012). At the present time, it is unclear how AtGRP7 and 8 affect cold stress response via the splicing process, including their targets. AtGRP7 has been reported to function as an RNA chaperone which unwinds RNA secondary structure to rescue an *E. coli* cold-sensitive strain or a tran-

scriptional anti-termination strain (Kim et al. 2007b; Kwak et al. 2011).

SR proteins are one of the key regulators for normal or alternative splicing events. SR proteins consist of a N-terminal RRM-type RNA-binding domain and a C-terminal serine-/arginine-rich domain which function to recognize target pre-mRNA and to facilitate protein-protein interactions for recruiting the core-spliceosome complex, respectively. In addition, SR proteins also function for recognizing splice sites to regulate alternative splicing of target pre-mRNAs, as well as glycine-rich RNA-binding proteins (GRPs), such as AtGRP7 and 8 (Duque 2011; Reddy et al. 2013). In addition, Palusa et al. (2007) reported that pre-mRNAs of the SR proteins are also alternatively spliced and half of them were degraded by NMD (Palusa et al. 2007). In order to determine whether splice variant mRNA of SR proteins were degraded or translated, polysomal association of splice variant mRNAs was analyzed under cold stress conditions (Palusa and Reddy 2015). Functional full length of *SR30*, *34*, and *34a* mRNAs were associated with polysomes in cold stress, while splicing component 35 kDa (SC35)-like (SCL) 33 was not enriched in polysomal fraction. These data indicated that SR30, 34, and 34a proteins function under cold stress and SCL33 is downregulated during the cold stress response. Although some members of splice variants of SR protein genes were associated with polysomes, splice variant forms of *SR30*, *34*, *34a*, and *SCL33* were not contained in the polysomal fraction and degraded by the NMD pathway. Thus, important correct SR proteins are translated and function under cold stress response to regulate the alternative splicing of target genes.

RS40 is a SR protein (Palusa et al. 2007) that interacts with high osmotic stress gene expression 5 (HOS5) and responds to osmotic stress (Chen et al. 2013b, 2015). The pre-mRNA of *RS40* is alternatively spliced which results in a significant enrichment of the full-protein containing form and a reduction of other alternatively spliced variants (Palusa et al. 2007). Although it is not reported, it seems that RS40 is fully functional under cold stress conditions. Additional studies are necessary to determine the function and target pre-mRNAs of *RS40* under cold stress conditions.

During the response to abiotic stress in plants, circadian clock and related gene regulations are also affected by alternative splicing (James et al. 2012; Staiger and Brown 2013; Seo and Mas 2015; Noren et al. 2016). Under cold stress conditions, *late elongated hypocotyl* (*LHY*), *pseudo-response regulator* (*PRR*) *7*, *PRR5*, and *timing of cab* (*TOC*) *1* generated unproductive isoforms and reduced the amounts of these functional proteins as a result of alternative splicing. In addition, a double mutant plant of *circadian clock-associated* (*CCA*) *1* and *LHY* genes exhibited high freezing tolerance (Dong et al. 2011). The triple mutants of *prr9*, *prr7*, and *prr5* have also been reported to exhibit cold stress tolerance (Nakamichi et al. 2009). Importantly, DREB/CBF genes are regulated by PRR9, PRR7, and PRR5, and *COR15A* and *RD29A* are also regulated by the circadian clock (Shinozaki and Yamaguchi-Shinozaki 2000; Msanne et al. 2011; Nakamichi et al. 2012; Liu et al. 2013). Collectively, these results indicated that circadian clock regulation by alternative splicing is an important component of the cold stress response in plants.

2.3.3 Quality Control System of mRNA

During the multiple steps of the mRNA maturation processes, it is inevitable that errors and mistakes may occur. Thus, RNA quality control is critical biological process which evolved to remediate these errors within mRNAs and to ultimately control the level of the correct forms of mature mRNAs prior to subsequent process of protein translation (Nicholson et al. 2010; Shaul 2015; Liu and Chen 2016). In yeast and humans, after the mRNA maturation step, correct forms of mRNAs are translated into proteins, and aberrant mRNAs are degraded. This elimination of aberrant mRNAs is regulated by mRNA surveillance mechanisms, such as nonsense-mediated decay (NMD), nonstop decay (NSD), and no-Go decay

(NGD) (Chiba and Green 2009; Shoemaker and Green 2012; Inada 2017). NMD is the most well-characterized system, which recognizes aberrant premature termination codons (PTCs) in mRNAs in order to avoid the production of truncated proteins or proteins lacking important functional domains (Kervestin and Jacobson 2012; Peccarelli and Kebaara 2014). NSD is the system which functions to recognize nonstop mRNAs, and NGD is a mechanism which specifically functions to repair stalling ribosomal proteins that are bound for aberrant mRNAs that prevent effective translation elongation (Shoemaker and Green 2012; Inada 2017). To date, two of these quality control systems (NSD and NGD), or similar degradation pathways, have not yet been identified in plants (Chiba and Green 2009).

As described in a previous section, alternative splicing results in exon skipping, intron retention, and the production of other variant isoforms, resulting in premature termination codons (PTC) of mRNAs. It is likely that the PTC-containing forms of mRNAs are targets for the NMD pathway, which has also been identified in plants (Shaul 2015).

Although there are some mechanistic differences of NMD between yeast, mammals, and plants, several homologous genes and proteins that are associated with NMD complexes have been identified in plants, such as up-frameshift (UPF), suppressor with morphogenetic effect on genitalia (SMG), and exon junction complex (EJC) (Riehs et al. 2008; Shi et al. 2012; Lloyd and Davies 2013; Nyiko et al. 2013). However, several other components of the NMD complex have not yet been identified in plants. Further functional analyses are warranted and necessary to identify and characterize the function of NMD and its physiological significance in plants.

Alternative splicing has been previously reported to increase during stress responses, and NMD has been well described to function together with alternative splicing under abiotic stress conditions in plants (Filichkin et al. 2010; Dubrovina et al. 2013; Leviatan et al. 2013; Staiger and Brown 2013; Kwon et al. 2014). In plants, exposure to cold stress increases alternative splicing, and PTC-containing aberrant mRNAs should be degraded by NMD. However, if NMD is impaired, tolerance to low-temperature stress is impaired. Further information is necessary to fully characterize these responses and their association to cold stress tolerance.

According to analyses using the *upf3* mutant, which is a major component of NMD, alternative spliced target genes of NMD and the level of their mature mRNAs were not increased in the mutant (Leviatan et al. 2013). In this case, these data suggest that NMD does not strongly affect transcripts. However, cold-inducible alternative splicing, which occurs in most genes (approximately 74%), produces some mRNA lacking functional domains, resulting in the production of new variations of transcripts that may play important roles in the response of plants to cold. Further analyses, such as global protein profiling, are necessary to reveal the functions of NMD and the expression pattern of these new types of transcripts.

2.4 mRNA Degradation

mRNA degradation is also an essential step which functions to regulate the expression levels of mRNAs and proteins (Belostotsky and Sieburth 2009; Xu and Chua 2011; Zhang and Guo 2017). The accumulation of mRNAs is determined by the balance of transcription and degradation speeds (Perez-Ortin et al. 2007). There are several categories for the accumulation of mRNAs:

1. Transcription is activated and is faster than the speed of degradation.
2. Degradation is suppressed and is slower than the speed of transcription; these types of mRNA accumulate in the cell.
3. Degradation is activated and is faster than transcription.
4. Transcription is suppressed and is slower than the speed of degradation; these types of mRNAs are reduced in quantity.

It is well known that gene and protein expression patterns are not identical, meaning that the levels

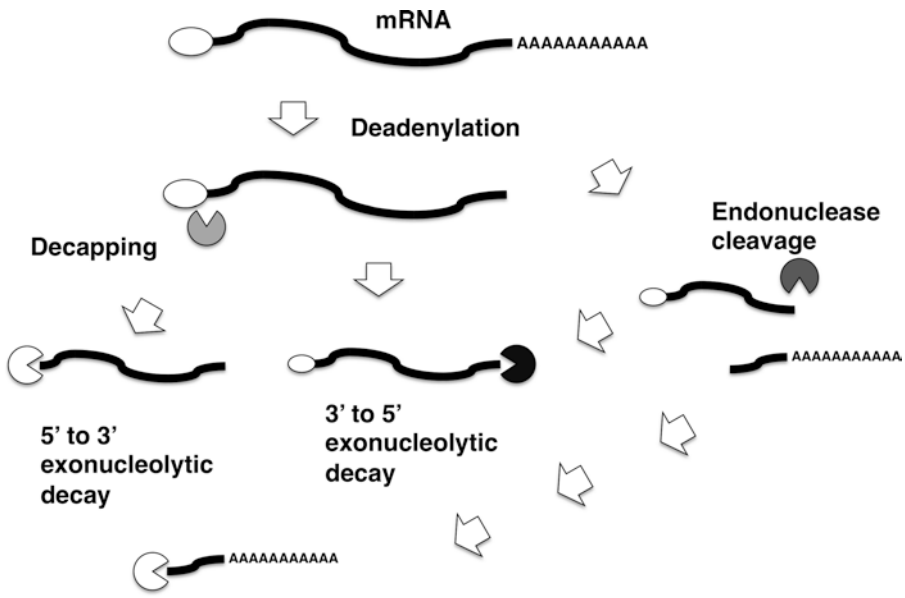

Fig. 2.4 Schematic illustration of the mRNA degradation pathway. After deadenylation, the m7G-cap structure is removed by the decapping enzyme. Uncapped mRNA is degraded in the 5′–3′ direction by the 5′–3′ exonuclease. Deadenylated mRNA is also degraded by a 3′–5′ exonuclease. Endonuclease-cleaved fragments of mRNAs are also degraded by the 5′–3′ or 3′–5′ exonucleolytic decay pathway

of transcribed mRNAs do not exactly correlate to the levels of the corresponding translated proteins. The disparity in the transcript to protein levels is primarily due to posttranscriptional effects, such as the degradation of mRNAs, or translational regulation, especially under stress conditions or during the response to changes in the environment (Kawaguchi et al. 2004; Pierrat et al. 2007; Hershey et al. 2012; Nakaminami et al. 2014). In addition, nontranslating mRNAs are likely degraded in messenger ribonucleoprotein (mRNP) granules, which are also known as processing bodies (PBs) in the cytosol (Bailey-Serres et al. 2009; Xu and Chua 2011).

The mechanisms associated with the degradation of mature mRNAs have been well-studied (Chiba and Green 2009). The first step (deadenylation) involves the removal of the poly(A) tail, which is then followed by decapping. This process results in the cleavage of mRNA in the middle of the molecule or degradation from the 3′ side (Fig. 2.4). This degradation system and components of the degradation complex have been identified and studied in plants.

When plants are exposed to cold stress, enzymatic activity, reaction speeds, and regulation mechanisms are reduced (Chiba et al. 2013). Selective degradation and transcription systems function to determine the level of mRNAs in response to cold stress. There have been several published reports pertaining to the degradation of mRNA in relation to cold stress tolerance and responses. In this section, the relationships of mRNA metabolism to cold stress responses in plants are summarized.

2.4.1 Deadenylation

Deadenylation of mRNAs is an essential process which marks the first step for initiating the degradation of mRNAs. The length of the poly(A) tail is an important factor which determines the stability of mRNA and also affects translational efficiency (Wiederhold and Passmore 2010; Weill et al. 2012). In plants, deadenylation is mediated by two types of enzymes (nucleases and deadenylases). Specifically, plants contain the poly(A)

nuclease (PAN) 2-PAN3 complex, poly(A)-specific ribonuclease (PARN), and poly(A) polymerases (PAP) and deadenylases such as carbon catabolite repressor4 (CCR4)/Ccr4-associated factor1 (CAF1)/negative on TATA (NOT) complex (Walley et al. 2010; Abbasi et al. 2013; Hirayama et al. 2013). At first, nucleases, such as the PAN2-PAN3 complexes, remove approximately 50–110 nucleotides of the poly(A) tail, and the CCR4/CAF1/NOT complex subsequently removes the majority of the remainder of the tail. PARN is localized in cytosol or mitochondria, and under stress conditions, the CCR4 complex is localized in processing bodies (Miller and Reese 2012; Hirayama et al. 2013; Suzuki et al. 2015).

Although it has not been reported that this deadenylation process is involved in cold stress responses, the PARN, CCR4, CAF1, and NOT complexes have been associated to other stresses such as defense response, salinity, and osmotic stresses (Nishimura et al. 2005, 2009; Liang et al. 2009; Walley et al. 2010). In these studies, CCR4 and CAF1 were shown to function in the deadenylation process in vivo. In addition, the target mRNAs of CCR4 and CAF1 are related to biotic and abiotic stress responses, suggesting that deadenylation is functionally important for stress responses in plants.

2.4.2 Decapping

Decapping, which is characterized by the removal of 7-methylguanosine 5′-diphosphate (m7G-cap), is the critical step for mRNA decay pathways and is mediated by decapping enzymes, such as decapping proteins (DCPs) and varicose (VCS), DEAD-box helicase homolog (DHH) 1, protein associated with topoisomerase II (PAT1), and the Sm-like protein (LSM) 1–7 complex (Xu et al. 2006; Goeres et al. 2007; Parker and Sheth 2007; Franks and Lykke-Andersen 2008; Xu and Chua 2009). In plants, these decapping enzymes are also localized in processing bodies (Weber et al. 2008). Decapping of mRNAs is important not only for plant development but also for stress responses (Goeres et al. 2007; Xu and Chua 2009, 2012). Recent studies have elucidated the relationship between mRNA decapping and cold stress response in plants.

In *Arabidopsis*, decapping proteins 1 and 2 (DCP1 and DCP2), which are essential enzymes for decapping, are localized in processing bodies. Under cold stress conditions, the levels of the DCP1 protein do not increase, but DCP1 forms granules in the cytosol. These data suggest that DCP1 plays an important role in the response of plants to cold stress (Motomura et al. 2015). Under normal or heat stress conditions, DCP1 co-localizes with DCP2. In contrast, however, only DCP1 bodies increase during periods of cold stress. These observations support the hypothesis that DCP1 has functional roles in both the degradation and stabilization of mRNAs, since DCP2 is a core subunit for mRNA decay and DCP1 is an auxiliary subunit that functions to recognize specific mRNAs (Xu et al. 2006; Deshmukh et al. 2008; Chang et al. 2014).

Sm-like protein (LSM) 1–7 complex is a decapping activator that was identified in plants and is localized in processing bodies in the cytosol (Perea-Resa et al. 2012). This complex recognizes mRNAs that are targeted for degradation and transports them to processing bodies to promote decapping. When double mutant plants of *lsm1a* and *lsm1b* were examined for freezing tolerance, cold-acclimated *lsm1a lsm1b* mutants exhibited high levels of freezing tolerance, whereas non-acclimated plants exhibited the same level of tolerance as wild-type plants (Perea-Resa et al. 2016). According to transcriptome analyses by RNA sequencing, many cold-inducible genes, such as *late embryogenesis abundant proteins* (*LEAs*), *zinc finger of Arabidopsis thaliana* (*ZAT*) *transcription factors*, and downstream genes related to cold acclimation, were upregulated in the mutant under cold stress conditions. These aforementioned genes are the potential targets of the LSM1-7 complex and should be fine-tuned by degradation. However, in the mutant plants, these cold acclimation-related genes were overaccumulated and resulted in the acquisition of a higher freezing tolerance phenotype than wild-type plants only after cold acclimation.

In *Brachypodium distachyon*, a global analysis of uncapped mRNAs, which is a decapped or deadenylated target mRNA analysis, revealed four types of target mRNAs that are categorized by stability and the expression pattern of mRNAs and degraded mRNAs (Zhang et al. 2013). Under cold stress conditions, it appears that cold-responsive mRNA tends to be degraded and translation-related genes, such as encoding ribosomal-related proteins, were not degraded and are more stable. These observations suggest that responsive genes need to be fine-tuned by degradation and that the stabilization of translational control is important for the cold stress responses. In addition, under cold stress conditions, the degradation target genes for decapping and deadenylation were significantly increased in relative comparison with NMD or exosome pathways, indicating that decapping and deadenylation are important for cold stress responses.

2.4.3 mRNA Degradation by Nuclease and Exosome

There are two types of mRNA degradation pathways that are governed by exoribonucleases. The first is a 5′–3′ mRNA degradation that is mediated by 5′–3′ exoribonuclease (XRN), and the second is a 3′–5′ directional degradation that is driven by an exosome complex (Fig. 2.4) (Kumakura et al. 2013; Nagarajan et al. 2013). These decay reactions also occur in processing bodies (Weber et al. 2008; Chiba and Green 2009). Several reports have demonstrated that mRNA degradation mediated by these exoribonucleases affects plant abiotic stress responses (Estavillo et al. 2011; Merret et al. 2013; Nguyen et al. 2015). However, there are almost no reports pertaining to these degradation enzymes in relation to cold stress responses and only those which study the relationship between nucleases and plant development (Chekanova et al. 2000; Hirsch et al. 2011; Rymarquis et al. 2011; Kumakura et al. 2013).

Although a relationship of these exoribonucleases has not yet been described for cold or freezing tolerance yet, a promoting factor for exoribonuclease activity has been reported to function in the cold stress response in plants. Specifically, FIERY1 (FRY1) is involved in chilling stress by mediating the dephosphorylation of 3′-phosphoadenosine 5′-phosphate (PAP) into adenosine-5′-phosphate (AMP) and inorganic phosphate (Pi), resulting in the promotion of XRN activity (Xiong et al. 2004). PAP inhibits XRN activity, and in the *fry1* mutant, PAP accumulates and results in a chilling-sensitive phenotype. Taken together, these data suggest that XRN activity might be important for the acquisition of chilling tolerance in plants.

2.5 RNA Stabilization

In order to characterize the dynamics of mRNA target degradation, mRNA decay, steady-state levels, and mRNA stabilization need to be considered as well. When the stabilization of mRNA was analyzed simultaneously, destabilized mRNA targets are also identified since the analysis of mRNA stabilization/decay is not only for determining the degraded targets of mRNAs but also steady-state level or stabilized mRNAs, which are not degraded mRNAs. That affects the final level of mRNAs and important as well as transcriptional regulation. In plants, the relationship of mRNA stability and degradation has been documented in relation to cold stress, and overviews of these processes are provided in several studies (Chiba et al. 2013; Zhang et al. 2013; Arae et al. 2017). As described in the decapping section (2.4.2 decapping), analysis of uncapped mRNA in *Brachypodium distachyon* revealed that cold-responsive mRNAs showed a tendency to be degraded, whereas translation-related genes were stabilized under cold stress conditions (Zhang et al. 2013). This analysis provided direct evidence to show that the selective control of mRNA degradation and stabilization is a key regulatory component in the cold stress response.

In *Arabidopsis*, recent analyses characterized mRNA decay during cold stress conditions on a global scale. Specifically, combined analyses of mRNA half-life measurements and mRNA expression levels were characterized under cold

stress conditions (Chiba et al. 2013). According to classification analyses, genes corresponding to many transcription factors and other factors related to plant growth and development, such as cell cycle-related genes, exhibited reduced mRNA levels. However, despite the fact that their transcript levels were not accumulated, the genes were stabilized. Under cold stress conditions, plants exhibit a cessation or reduction of growth. Expression patterns of growth and developmental genes are in accordance to this response, indicating that the suppression of these genes is due to transcriptional reduction and not due to changes in the stability of their mRNAs under cold stress conditions. In contrast, various biotic and abiotic stress-responsive genes become destabilized under stress conditions. However, despite the fact that these genes tend to degrade under cold stress conditions, they still accumulate during the cold stress response. For example, cold-responsive genes, such as *COR15A*, are destabilized, and their mRNAs still accumulate in response to cold stress. These observations suggest that this gene category needs to be quickly degraded in order to enable a rapid response for exposure to environmental stress, since mRNA levels are easily controlled by its shortened half-life. These aforementioned changes of mRNA accumulation and degradation rates appear contradictory to one another. These observations are also consistent with findings in *Brachypodium distachyon*, indicating that plants have evolved complex mechanisms for regulating mRNA expression at the transcriptional or posttranscriptional levels (Zhang et al. 2013; Arae et al. 2017).

In a study by Nakaminami et al. (2014), they reported another mechanism of mRNA stabilization under stress conditions and clarified the correlation between mRNA and protein expression. Specifically, differential expression analyses were performed between mRNAs and proteins during the cold stress response in plants. As previously described, the expression of mRNAs and proteins are not consistent with each other due to regulation at the posttranscriptional or translational levels. The authors hypothesized that under cold stress conditions, some mRNAs are expressed but are not simultaneously translated, such as transcripts that are protected and stabilized during cold acclimation and de-acclimation steps. According to comprehensive comparative expression analyses of mRNAs and proteins during cold stress conditions, three types of mRNA/protein relationships were identified: (A) both mRNAs and proteins are expressed during the cold acclimation step; (B) mRNAs are expressed during cold acclimation, but corresponding proteins are expressed during de-acclimation to cold stress; and (C) both mRNAs and proteins are expressed during the de-acclimation process (Fig. 2.5). The mRNAs/proteins categorized in (A) function for obtaining cold acclimation and (C) mRNAs/proteins function for cold de-acclimation, respectively. With respect to category (B), mRNAs/proteins are stabilized mRNA targets, whose protein translation is terminated during the cold acclimation step, but the translation is initiated early within the de-acclimation step. During this translation process, de novo mRNA synthesis is not required and, instead, utilizes reserved mRNAs which are transcribed during the cold acclimation step, indicating that that this early protein expression enables plants to rapidly respond during the transition from a cold-acclimated state to de-acclimation. Type (B) mRNAs/proteins contain translation-related proteins, such as ribosomal mRNA and eukaryotic translation initiation factors, suggesting that during the early de-acclimation step, plants require rapid protein expression for adapting to environmental changes. Consequently, they produce translational components without mRNA expression. This result is consistent with those described by Zhang et al. (2013) where stabilized targets were found to correspond to genes encoding ribosomal-related proteins (Zhang et al. 2013). Collectively, these two analyses suggest that plants have evolved complex and programmed mechanisms to enable dynamic responses to cold stress.

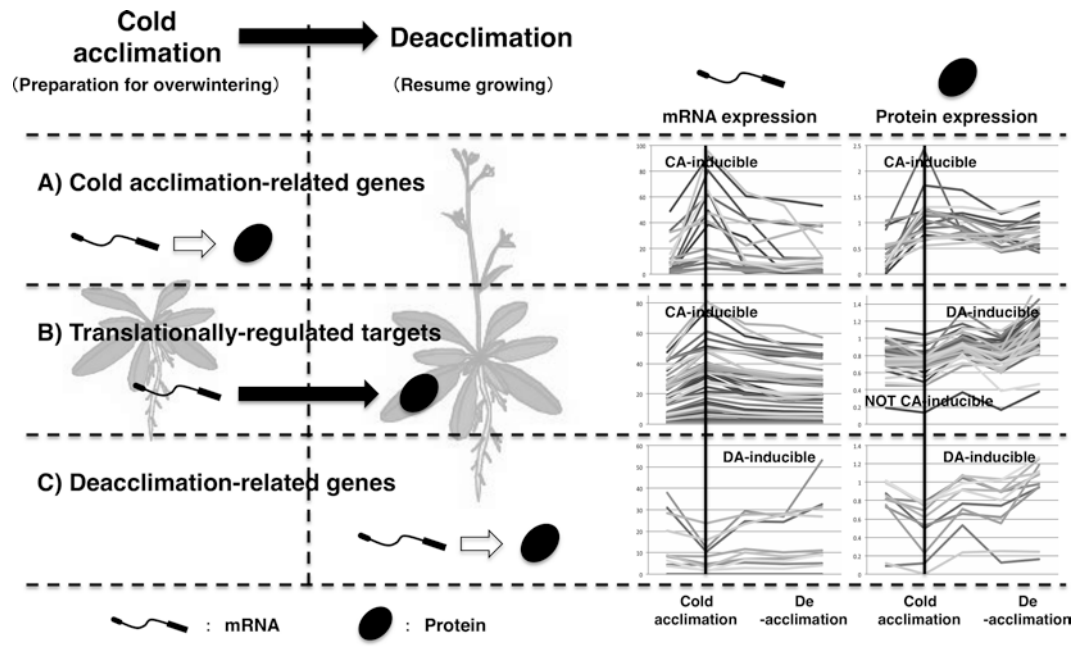

Fig. 2.5 Comparative analysis of mRNAs and corresponding proteins during cold acclimation and de-acclimation in *Arabidopsis*. (Left) Illustration of mRNAs/proteins is categorized into three types of clusters by expression patterns, (**a**) cold acclimation-related genes, (**b**) translationally regulated stabilized targets, and (**c**) de-acclimation-related genes. (Right) mRNA and protein expression pattern of these categorized clusters. Solid vertical lines indicate the time point of maximal cold acclimation (2° for 7 days), and the right side of the line in the graph represents expression profiles after a de-acclimation treatment

2.6 Stress Granules and Processing Bodies

In the cytosol, messenger ribonucleoprotein (mRNP) granules are involved in the regulation of mRNA, processing bodies (PBs) primarily function for mRNA decay, and stress granules (SGs) are involved in the stabilization of mRNA (Fig. 2.2) (Bailey-Serres et al. 2009; Xu and Chua 2011). As described in previous sections, PBs contain several mRNA degradation enzymes, such as decapping enzymes and nucleases. Additionally, PBs also function in the repression of translation; thus, ribosomal- and translation-related proteins are generally excluded from PBs, with the exception of eukaryotic translation initiation factor 4E (eIF4E) since eFI4E is likely associated with nontranslating mRNAs (Andrei et al. 2005; Ferraiuolo et al. 2005). In addition to the degradation of normal mRNAs, aberrant mRNAs, which are identified by the quality control system, are also degraded within PBs.

SGs are consist of RNA-binding proteins and nontranslating mRNAs, which are stabilized and protected by degradation. In addition, SGs also contain translation initiation components, such as ribosomal 40s subunit and translation initiation factors; however, these are in an inactive form, and the translation of target mRNAs is suppressed (Decker and Parker 2012). Oligouridylate-binding proteins (UBPs) are essential proteins for the formation of SGs. Although these proteins have been described to function in relation to various abiotic stresses in plants, there are no reports describing a functional role specifically for cold stress response or tolerance to date (Sorenson and Bailey-Serres 2014; Nguyen et al. 2016).

Under abiotic stress conditions, PBs and SGs appear and increase the size of granules (Weber et al. 2008; Sorenson and Bailey-Serres 2014; Motomura et al. 2015; Nguyen et al. 2016). Within these cytosolic granules, several steps of posttranscriptional regulation and mRNA regula-

tion occur. PBs and SGs co-localize with one another and are probably close to active translational complexes, polysomes. Although there have been several recent studies which have described the detailed function of PBs and SGs and their components, few reports, however, describe the relationship between these granules and cold stress response in plants.

Processing bodies, stress granules, and polysomal complexes consist of messenger ribonucleoprotein (mRNP) in the cytosol and also contain many RNA-binding proteins. After transcription, mRNAs are not alone and are always recognized and bound by several types of RNA-binding proteins. These mRNPs are specifically localized in the nucleus and cytosol, processing bodies, and stress granules and also move with target mRNAs. Several reports have described a functional role for RRM-type or cold shock domain type of RNA-binding proteins in abiotic stress responses, especially cold stress tolerance, and are summarized in review papers (Nakaminami et al. 2012; Lee and Kang 2016). These RNA-binding proteins, such as cold shock proteins (CSPs), glycine-rich RNA-binding proteins (GRPs), and helicases, function as RNA chaperones. The chaperone activity functions to unwind the secondary structures of RNA and promote or affect mRNA stabilization or translation. At the present time, it is still unclear if these RNA-binding proteins function in stress granules or polysomes. Further functional analyses which aim to characterize these RNA-binding proteins and their direct mRNA targets will be essential to decipher their roles in RNA regulation at the posttranscriptional level.

2.7 Conclusions and Perspectives

Overwintering plants are capable of acquiring chilling or freezing tolerance due to a complex regulation of genes and proteins with specific functions related to the adaptation to cold stress conditions. In this review, specific emphasis was placed on the regulation of mRNA at the posttranscriptional level, occurring just after transcription and prior translation.

Figure 2.6 illustrates the summarized processes of transcription, posttranscriptional regulation, and translation that are affected by the cold stress response in plants. When non-acclimated plants are exposed to cold stress conditions, steady-state mRNA transcription and degradation occur, with deleterious effects on normal plant growth and development. During the progression from autumn to winter under natural conditions, herbaceous plants, such as *Arabidopsis*, are capable of perceiving reductions of temperature, which can serve as cues to increase freezing tolerance via the cold acclimation process. During the cold acclimation step, alternative splicing increases the incidence of premature termination codons (PTC), resulting in the formation of aberrant mRNA or truncated mRNAs which are then degraded in processing bodies (PBs). In addition to the degradation of aberrant or truncated mRNAs, mRNAs, which are supposed to be eliminated, are also degraded by PBs. Cold-responsive mRNAs should be translated into proteins and function for increasing freezing tolerance, while stabilized nontranslating mRNA targets are protected in stress granules (SGs). Notably, cold-responsive mRNAs tend to be degraded and destabilized, and subsets of mRNAs are degraded in PBs in order to regulate the final level of mRNAs for translation. During spring, plants sense the temperature upshift during the change of the season and initiate rapid translation without de novo mRNA synthesis, by utilizing mRNA templates that were stored in SGs from the cold acclimation step. These rapidly translated protein products are translational components and promote de novo transcription and translation that is required for reinitiating plant growth. Taken together, these observations suggest that plants have evolved complex regulated mechanisms to program the expression of mRNAs and proteins that are functionally involved in the perception of seasonal changes and for the cold stress response.

In response to abiotic stress, such as the cold stress response in plants, the majority of regulation occurs at the level of gene expression when

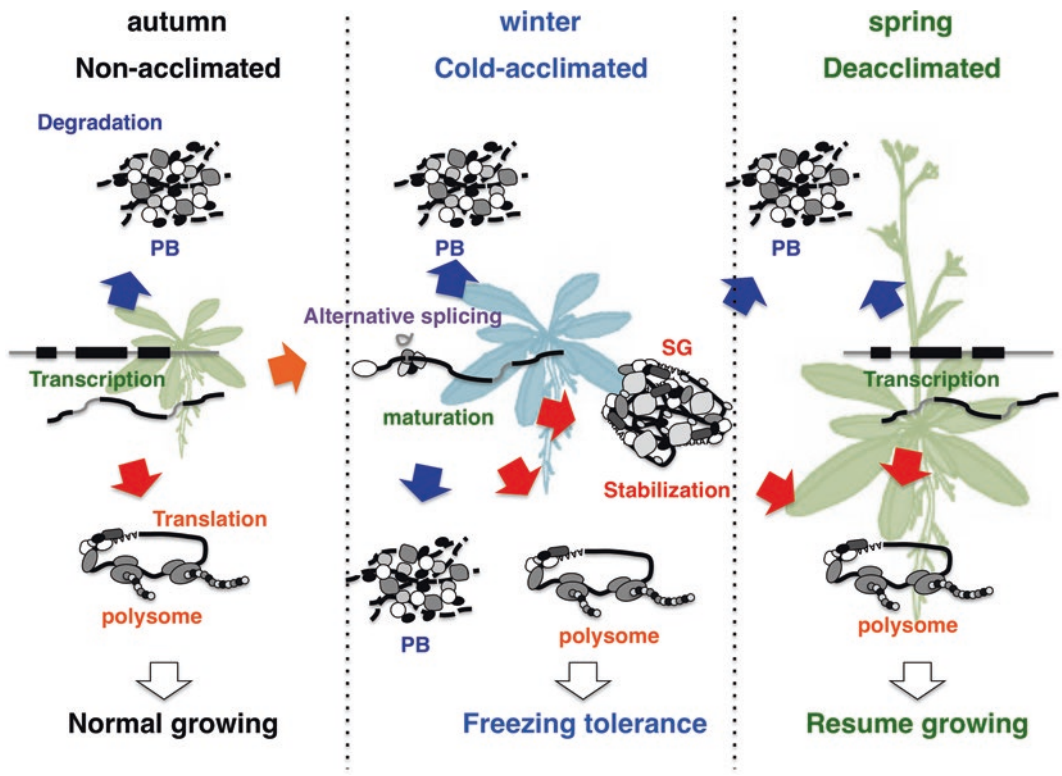

Fig. 2.6 Schematic illustration of posttranscriptional mRNA regulation during cold acclimation and deacclimation. Polysome indicates active translational complex; PB represents processing body involved in degradation. SG indicates stress granule related to stabilization

specific subsets of cold response mRNAs are induced. Under most circumstances, cold stress-responsive genes typically have very low to no expression during normal conditions, and dramatic fold changes of expression are induced in response to the exposure to cold stress. However, when expression levels of their corresponding proteins are measured, they are not completely consistent to each other, resulting from mRNA regulation at posttranscriptional or translational level.

According to analyses using several chilling- or freezing-sensitive mutants with impaired functionality in various steps of mRNA regulation, some unexplainable patterns of mRNA regulation were observed. For example, cold-responsive genes were upregulated in mutant plants, even if the plants exhibited a stress-sensitive phenotype. In the case of splicing mutants, cold-responsive genes overaccumulate, and the expected result of the mutant phenotype is cold tolerance. However, the mutant showed a sensitive phenotype since mis-spliced forms of mRNA targets resulted in the production of aberrant proteins, such as PTC-containing proteins or truncations within functional domains. It is plausible that this mis-splicing is the reason why the mutants exhibit altered tolerance to the stress conditions, since proper splicing is crucial for the production of functional proteins that would enable wild-type plants to respond to exposure to stress conditions. In another example of an unexplainable observation regarding mRNA regulation and the cold stress response, global mRNA degradation analyses revealed that cold-inducible genes exhibited a tendency to degrade under stress conditions (destabilized transcript category), even if the corresponding mRNAs were accumulated during cold stress conditions. It is unclear and difficult to explain why these changes of mRNA

accumulation and degradation appear to exhibit opposite patterns to one another. It is possible that these cold-responsive genes might rapidly degrade to conserve energy since the translation step consumes the largest amount of energy among all cellular processes. In addition, this tendency of destabilized mRNAs does not affect the sufficient accumulation of mRNAs and is most likely important for fine-tuning and determining final mRNA and protein level or suppressing the overaccumulation of mRNAs in order to enable a response to rapid changes within the environment.

Plants have evolved complex mechanisms for regulating mRNA expression at the transcriptional or posttranscriptional levels. In order to explain the phenotypes associated with the response to cold stress, and to fully understand the detailed mechanisms involved with the response, it is important to clarify the structure of correct or aberrant mRNA isoforms and/or corresponding protein expression or activity. Recently, expression analysis technologies, such as next-generation sequencing, have greatly improved our capabilities to fully characterize and understand the expression of mRNAs at the structural level. Although it is challenging, comprehensive proteomic analyses have also become greatly improved and now make it possible to clarify the mechanism of posttranscriptional or translational regulation with comparative analyses at the gene and protein level. Studies which focus on the fine-tuning of posttranscriptional mRNA regulation are essential in order to fully understand the cold stress response in plants. These precise mRNA regulatory mechanisms might be provided a clue to improve the cold stress tolerance of crops in the future.

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The Mechanism Enabling Hibernation in Mammals

3

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Abstract

Some rodents including squirrels and hamsters undergo hibernation. During hibernation, body temperature drops to only a few degrees above ambient temperature. The suppression of whole-body energy expenditure is associated with regulated, but not passive, reduction of cellular metabolism. The heart retains the ability to beat constantly, although body temperature drops to less than 10 °C during hibernation. Cardiac myocytes of hibernating mammals are characterized by reduced Ca^{2+} entry into the cell membrane and a concomitant enhancement of Ca^{2+} release from and reuptake by the sarcoplasmic reticulum. These adaptive changes would help in preventing excessive Ca^{2+} entry and its overload and in maintaining the resting levels of intracellular Ca^{2+}. Adaptive changes in gene expression in the heart prior to hibernation may be indispensable for acquiring cold resistance. In addition, protective effects of cold-shock proteins are thought to have an important role. We recently reported the unique expression pattern of cold-inducible RNA-binding protein (CIRP) in the hearts of hibernating hamsters. The CIRP mRNA is constitutively expressed in the heart of a nonhibernating euthermic hamster with several different forms probably due to alternative splicing. The short product contained the complete open reading frame for full-length CIRP, while the long product had inserted sequences containing a stop codon, suggesting production of a C-terminal deletion isoform of CIRP. In contrast to nonhibernating hamsters, only the short product was found in hibernating animals. Thus, these results indicate that CIRP expression in the hamster heart is regulated at the level of alternative splicing, which would permit a rapid increment of functional CIRP when entering hibernation. We will summarize the current understanding of the cold-resistant property of the heart in hibernating animals.

Keywords

Hibernation · Cold-shock protein · Hypothermia

Abbreviations

CIRP	Cold-inducing RNA-binding protein
CNS	The central nervous system
ECG	Electrocardiograms
HNF	Hepatocyte nuclear factor

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HP	Hibernation-specific protein
ICV	Intracerebroventricular
RBM3	RNA-binding motif 3
SERCA2	asarco(endo)plasmic reticulum Ca^{2+}-ATPase 2a

3.1 Hibernation of Mammals

Most mammals have the ability to maintain their body temperature within a narrow range even in a cold environment. In a cold environment, thermoregulatory responses to minimize heat loss (e.g., peripheral vasoconstriction and piloerection) are evoked (Tansey and Johnson 2015). In addition, heat-producing responses in skeletal muscles (shivering thermogenesis) and in brown adipose tissue (non-shivering or metabolic thermogenesis) are activated, and thereby a drop in body temperature is prevented (Tansey and Johnson 2015). If the body temperature of homotherms drops extremely, they cannot survive because the heart cannot keep beating, and organs fall into ischemia (Ivanov 2000).

On the other hand, several mammalian species undergo hibernation (Carey et al. 2003; Ruf and Geiser 2015) (see Table 3.1). During hibernation, body temperature drops to only a few degrees above ambient temperature (Carey et al. 2003; Ruf and Geiser 2015). Hibernating animals stay unmoving and usually show a curly shape to minimize heat dissipation from the body surface (Fig. 3.1). The hypothermic condition of mammalian hibernators is fundamentally different from that of poikilotherms (amphibians and reptiles). The body temperature of poikilothermic animals directly correlates with changes in ambient temperature due to a lack of efficacious mechanisms for maintaining body temperature (Jackson and Ultsch 2010; Malan 2014). As a result, body temperature drops passively in response to a decrease in ambient temperature. In contrast, mammalian hibernators possess a thermoregulatory mechanism similar to that of nonhibernators, and they can control their body temperature in a nonhibernating state despite exposure to a wide range of surrounding temperatures (Carey et al. 2003; Ruf and Geiser 2015). Even in an extremely cold environment, they do not necessarily undergo hibernation if enough food is available. Furthermore, mammalian hibernators do not always continue in a hibernating condition throughout winter; they sometimes interrupt hibernation and spontaneously recover their body temperature even though they are consistently exposed to a cold environment (Carey et al. 2003). This behavior provides further evidence for the notion that hypothermia during mammalian hibernation is actively induced, since passively induced hypothermia may not recover unless ambient temperature is increased. Thus, hibernation of mammals is considered to be an adaptive strategy to survive in a severe environment during winter.

Table 3.1 Hibernating mammals

Order	Species	Body temperature in hibernation (°C)
Monotremata	Echidna	4
Marsupialia	Pygmy possum, feathertail glider, Chiloe opossum	1.3–7.1
Eulipotyphla	Hedgehog	1–9.7
Afrosoricida	Tenrec	8.6–15
Chiroptera	Bat	−2 to 13.9
Primates	Lemur	6.5–9.3
Carnivora	Badger, bear	28–32.5
Rodentia	Prairie dog, marmot, woodchuck, ground squirrel, chipmunk, pocket mouse, kangaroo mouse, hamster, jerboa, dormouse	−2.9 to 15

Ruf and Geiser (2015)

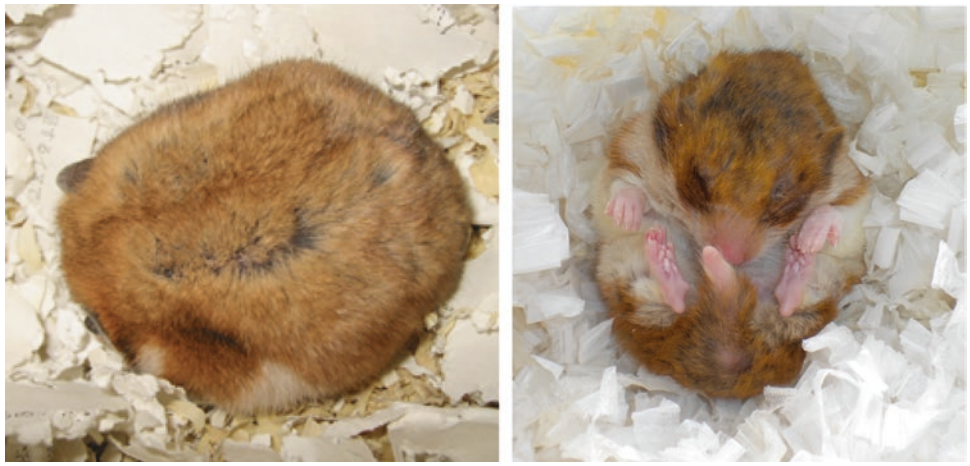

Fig. 3.1 Hibernating hamster. Pictures show curly shape that is usually observed during hibernation in Syrian hamsters

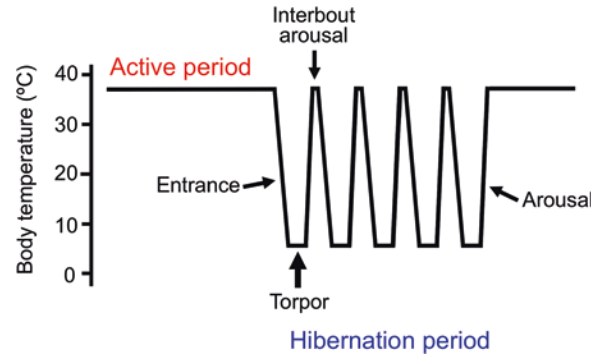

Fig. 3.2 A schema of body temperature during hibernation

3.2 Variation of Hibernation

During hibernation, the degree of body temperature reduction and duration of the hypothermic state vary widely among animal species (Carey et al. 2003; Ruf and Geiser 2015). In black bears, the body temperature during hibernation is around 33 °C, which is much higher than that in small hibernators (Carey et al. 2003; Ruf and Geiser 2015). In contrast, the body temperature of arctic ground squirrels drops to as low as −3 °C during hibernation (Barnes 1989). Several mammalian species undergo daily torpor, in which duration of the hypothermic state is less than 24 h (Breukelen and Martin 2015). In daily torpor, reduction of body temperature is relatively moderate compared with that in deep hibernation. Exceptionally, it has been reported that body temperature of the rock elephant shrew (*Elephantulus myurus*) reached 5–10 °C during daily torpor (Mzilikazi et al. 2002). Some species including tenrec and mouse lemurs adopt either daily torpor or hibernation depending on the ambient temperature (Lovegrove and Génin 2008; Kobbe and Dausmann 2009; Kobbe et al. 2011). A typical deep hibernation is characterized by extended duration of torpor bouts. As shown in Fig. 3.2, the hypothermic state during deep hibernation is interrupted by periods of arousals to euthermia, so-called interbout arousals. The duration of torpor bouts is from a few days to up to 5 weeks. The interbout arousals are maintained for 12–24 h before reentry into torpor (Carey et al. 2003). The periodic hibernation-arousal cycles suggest that the central nervous system (CNS) is continuously operated even at a low temperature during hibernation.

Table 3.2 Physiological parameters in active and hibernating hamsters ($n = 6$)

	Active control	Hibernation in summer	Hibernation in winter
Body temperature (°C)	35.2 ± 0.6	5.0 ± 0.9	5.5 ± 0.3
Heart rate (beats/min)	369 ± 13	15.8 ± 3.1	15.0 ± 2.7
Respiratory rate (breaths/min)	92.2 ± 8.5	2.3 ± 1.7	3.0 ± 1.4

It is known that seasonal hibernators, e.g., Richardson's ground squirrel (*Spermophilus richardsonii*) and Siberian chipmunk (*Tamias sibiricus asiatics*), rarely hibernate in summer even if they are placed in a cold condition (Kondo 1987). This suggests that the endogenous circannual rhythm plays a critical role in the induction of hibernation in seasonal hibernators. In contrast, hamsters hibernated even in summer when they were placed in a condition suitable for induction of hibernation (Miyazawa et al. 2008). No significant differences in parameters including body temperature, heart rate, respiratory rate (Table 3.2), and incidence of ECG abnormalities were found between hibernation in summer and that in winter (Miyazawa et al. 2008). Therefore, the endogenous circannual rhythm might only have a minor contribution, if any, to the induction of hibernation in this species. Of course, this does not necessarily rule out the possibility of involvement of the circannual rhythm in the induction of hibernation in hamsters. It may be appropriate to consider that the relative importance of endogenous and environmental factors varies among species and that this variation is a determinant for seasonal or nonseasonal hibernators.

3.3 Metabolic Regulation During Hibernation

It is generally accepted that the primary purpose of hibernation is to decrease metabolic activity, allowing energy expenditure to be balanced with reduced energy supply due to limited food availability during the winter season. For instance, the metabolic rate of the hibernating ground squirrel is reduced to less than 5% of that observed in the nonhibernating euthermic counterpart (Wang and Lee 1996). The suppression of whole-body energy expenditure is associated with regulated, but not passive, reduction of cellular metabolism. It has been demonstrated that a serine/threonine protein kinase, Akt (also known as protein kinase B), is inactivated by dephosphorylation in hibernating animal organs, typically in skeletal muscles and the liver (Abnous et al. 2008). Considering that Akt activation plays an important role in anabolic and catabolic responses in various cells, the dephosphorylation of Akt in hibernating animal cells would be suitable for a decrease in metabolic activity. Interestingly, the dephosphorylation is promoted immediately prior to entering hibernation (Hoehn et al. 2004). Accordingly, the reduction of cellular metabolism during hibernation does not arise as a consequence of lowered temperature (i.e., general suppression of enzyme activity). Rather, cellular metabolism is suppressed actively before entering hibernation, and this can therefore be a cause of decrease in body temperature. Consistent with this, Akt activity is increased during arousal from hibernation (Lee et al. 2002; Fleck and Carey 2005).

Some species do not feed during hibernation, whereas other species store food and feed during interbout arousals (Humphries et al. 2003; Geiser 2004). Regardless of these differences, hibernation in both groups of species can be considered as a fasting condition (Humphries et al. 2003). To tolerate the long-term fasting condition, major metabolic substrate switches from glucose to lipid occur during the hibernation period in ground squirrels and black bears (Serkova et al. 2007; Andrews et al. 2009) as evidenced by the fact that respiratory quotient values are about 0.7 in hibernating animals (Fedorov et al. 2009).

Global analysis of gene expression by using DNA microarrays would allow speculation regarding differences in metabolic conditions between hibernating hypothermic animals and active euthermic animals (Williams et al. 2005). In the liver of hibernating bears, expression levels of key glucogenic enzymes are increased,

whereas expression levels of glycolytic enzymes are decreased (Fedorov et al. 2009). A similar shift from glycolysis to gluconeogenesis was observed at the mRNA and protein levels in the liver of hibernating ground squirrels (Yan et al. 2008). These changes would contribute to the provision of glucose as an energy source for the brain and other tissues in fasting conditions during hibernation. Also, genes involved in cellular respiration are downregulated during hibernation (Williams et al. 2005; Yan et al. 2008; Fedorov et al. 2009). This is consistent with the reduced metabolic rate in hibernating animals (Carey et al. 2003). Reduction of gene expression for anabolic enzymes with concomitant induction of gene expression for catabolic enzymes is also the case in lipid metabolism. A coordinated induction of genes involved in fatty acid β-oxidation and downregulation of genes involved in lipid biosynthesis at transcriptional (Williams et al. 2005; Yan et al. 2008) and proteomic levels (Shao et al. 2010) have been shown in the livers of hibernating bears and ground squirrels. In contrast, genes involved in amino acid catabolism are downregulated during hibernation (Fedorov et al. 2009). Reduction of amino acid breakdown would be reasonable, since genes involved in protein biosynthesis in the liver and skeletal muscles are increased in this state (Fedorov et al. 2009). The enhanced protein biosynthesis is considered to be a molecular adaptation that contributes to the ability to reduce muscle atrophy over prolonged periods of immobility during hibernation.

3.4 Endogenous Regulators of Hibernation

3.4.1 Factors Related to Hibernation

Although the precise mechanism responsible for regulating hibernation remains unknown, a number of studies have revealed important factors controlling hibernation behavior. It has been demonstrated that adenosine acting through the adenosine A1-receptor in the CNS plays a key role in the entrance phase of hibernation in hamsters (Tamura et al. 2005). The importance of central adenosine is suggested by the fact that intracerebroventricular (ICV) injection of an adenosine A1-receptor antagonist to hamsters in the process of dropping body temperature inhibits entrance to hibernation (Tamura et al. 2005). The effect of adenosine would be related to hibernation onset but not to maintenance of a hypothermic condition, because decreased body temperature cannot be reversed in animals in which deep hypothermia has already been established (Tamura et al. 2005). Vice versa, ICV injection of an A1-receptor agonist to euthermic hamsters decreases body temperature (Miyazawa et al. 2008). In the CNS, adenosine acts as a neuromodulator, and the A1-receptor mediates the presynaptic inhibition of neurotransmission. Thus, activation of the A1-receptor would act as a suppressor of the thermoregulatory mechanism in the CNS. In accordance with this, it has been reported that activation of the A1-receptor promotes sedation and depression of locomotor activity (Radulovacki et al. 1982; Wauquier et al. 1987; Nikodijevic et al. 1991; Ticho and Radulovacki 1991; Malhotra and Gupta 1997).

Similar approaches to identify possible regulators of hibernation have revealed that opioid peptides such as β-endorphin and endomorphine in the hypothalamus are related to the maintenance phase via the μ1-opioid receptor in hamsters (Tamura et al. 2005) and via the δ-opioid receptor in ground squirrels (Yu and Cai 1993a, b). The contribution of opioid peptides to maintenance of the hypothermic state leads an interesting hypothesis that continuous release of opioid peptides during hypothermia may induce tolerance, and therefore hamsters cannot maintain hypothermia for a long time (Tamura et al. 2005). Although further study is needed to verify this hypothesis, it provides a rational explanation for the presence of energy-demanding interbout arousals.

Several lines of evidence suggest that histamine in the hippocampus is involved in the maintenance of hibernation. In general, histamine decreases sleep and promotes wakefulness (Nishino et al. 2001). However, infusion of hista-

mine into the dorsal hippocampus brings about prolonged duration of the torpor bout. This finding is also interesting since it supports the idea that hibernation is an arousal state distinct from any known euthermic arousal state, rather than being homologous to sleep (Kilduff et al. 1993).

The preferential use of lipids during hibernation seems to suggest that excessive fat accumulation is appropriate for entering hibernation. However, it has been demonstrated that a high body mass inhibits the induction of hibernation (Bieber et al. 2014; Zervanos et al. 2014). Conversely, a reduction of body mass triggers the entrance to hibernation in order to reduce the consumption of limited amounts of stored fat. Thus, the decision of whether or not to enter hibernation depends on the body mass and amount of fat deposits (Humphries et al. 2003; Chayama et al. 2016). One possible hormonal mediator that reflects the amount of fat deposits is leptin (Houseknecht et al. 1998). In line with this, a high circulating level of leptin negatively impacts the induction of hibernation. In little brown bats, dissociation of leptin secretion and adiposity is found during the pre-hibernation period, and the decreased leptin level in the absence of a decrease in body mass permits the entrance to hibernation (Kronfeld-Schor et al. 2000). Accordingly, leptin can be considered to be an important regulator of hibernation.

3.4.2 Hibernation-Specific Protein

Many studies have been conducted to identify factors responsible for hibernation (Wang et al. 1988; Shintani et al. 2005; Tamura et al. 2005, 2006, 2012; Kondo 2007; Chayama et al. 2016). The most typical factors that may play a role in physiological adaptation prior to the onset of hibernation are hibernation-specific proteins (HP), originally discovered in the chipmunk (*Tamias asiaticus*) in 1992 (Kondo and Kondo 1992). The protein identified in the plasma of the chipmunk is a 140-kDa protein complex that consists of four components: three highly homologous proteins (HP-20, HP-25 and HP-27) and a proteinase inhibitor (HP-55) (Kondo and Kondo 1992; Takamatsu et al. 1993). HP-20, HP-25, and HP-27 contain an N-terminal collagen-like domain and a C-terminal globular domain homologous to the complement C1q (Takamatsu et al. 1993). The proteins can be detected in the plasma of hibernators, but not in nonhibernators, including tree squirrels and rats (Kondo and Kondo 1992; Takamatsu et al. 1993). The lack of HP in tree squirrels (*Callosciurus caniceps*) is interesting because tree squirrels are a species closely related to chipmunks but do not undergo hibernation (Kojima et al. 2001). This provides support for the pivotal role of HP in hibernation.

The plasma level of the HP complex decreases markedly in hibernating chipmunks (Kondo and Kondo 1992; Takamatsu et al. 1993). Concomitantly, HP gene expression in the liver, in which HP is exclusively produced, is down-regulated (Takamatsu et al. 1993). However, reduction of the circulating HP complex level would not be totally dependent on reduced production in the liver. In contrast to the plasma level of the HP complex, the level of a heterotrimer composed of HP-20, HP-25, and HP-27 (called HP20c) is increased in the brain (Kondo et al. 2006). Therefore, transport to the brain is attributable to the reduced circulating level of the HP complex. The currently accepted mechanism for activation of HP involves dissociation of the HP complex to HP20c and HP-55. HP20c, being free from HP-55, can be actively transported to the brain, where it regulates brain functions for hibernation. In support of this model, a neutralizing antibody against HP20c decreases the duration of hibernation (Kondo et al. 2006). Furthermore, hibernation is never induced in animals lacking an increase in HP20c even in a cold environment (Kondo et al. 2006). The precise action of HP20c in regulation of hibernation remains to be elucidated.

Interestingly, HP gene expression levels in the liver, as well as plasma HP levels, show seasonal oscillations even when chipmunks are kept under a warm condition with a 12-h photoperiod (Kondo et al. 2006). This indicates that gene expression of HP is regulated by endogenous circannual rhythms, rather than environmental factors (Kondo et al. 2006). It has been demon-

strated that hepatocyte nuclear factor 4 (HNF-4) activates *HP-25* transcription (Kojima et al. 2000). In nonhibernating chipmunks, HNF-4 binds to the *HP-25* promoter, leading to *HP-25* transcriptional activation. On the other hand, small heterodimer partner (SHP), which is a negative regulator of HNF-4, is upregulated in the liver of hibernating chipmunks, resulting in the dissociation of HNF-4 from the *HP-25* promoter and the repression of *HP-25* gene transcription (Tsukamoto et al. 2017). Accordingly, SHP, which controls HNF-4 binding to the *HP-25* gene promoter, would be one of the key regulators of HP gene expression.

3.5 Regulation of Cardiac Function During Hibernation

3.5.1 Innate Characteristics of the Heart of Hibernators

Although heart rate in hibernating animals is dramatically lowered compared with that in euthermic counterparts, normal sinus rhythm is fundamentally maintained (Harris and Milsom 1995; Milsom et al. 1999; Mertens et al. 2008). This is in contrast to nonhibernating mammals, in which ventricular dysfunction and arrhythmias such as atrioventricular block and ventricular fibrillation develop when their body temperature drops to less than 20 °C (Johansson 1996; Fedorov et al. 2008). Contraction of cardiac muscle, analogous to that of skeletal muscle, is induced by intracellular Ca^{2+} transients (Kurihara 1994). Hence, a rise in intracellular Ca^{2+} concentration sufficient for inducing contraction is needed to maintain heart function at a cold temperature. In the rat myocardia, basal intracellular Ca^{2+} concentration, which is usually about 140 nM at 30–35 °C, is increased to 200–300 nM in response to a cold temperature around 10 °C (Liu et al. 1991; Wang and Zhou 1999). Such a rise in the basal Ca^{2+} concentration would negatively impact cardiac function, since it enhances basal tone, resulting in insufficient dilation during the diastolic filling period. Furthermore, a rise in the basal Ca^{2+} concentration also lowers the degree of Ca^{2+} increment even if the amplitude of the cytosolic Ca^{2+} transient remains similar. It is therefore considered that a rise in the basal Ca^{2+} concentration is an underlying basis for cardiac dysfunction at a cold temperature.

Interestingly, in the ground squirrel, a typical hibernator, it has been reported that the basal intracellular Ca^{2+} concentration of myocardia is not increased at a cold temperature (Wang et al. 2002). In addition, amplitude of the Ca^{2+} transient is increased at a cold temperature (Wang et al. 2002). In agreement with this, myocardial contractile force at a low temperature is greater than that at a temperature comparable to body temperature of the active state. The greater myocardial contractile force at a low temperature would be reasonable as a compensatory mechanism for the marked reduction of heart rate. The remarkable differences in Ca^{2+} dynamics between hibernators and nonhibernators suggest that an ability to maintain cardiac contractility under an extremely hypothermic condition can be recognized as an inherent feature of hibernators.

The cold-resistant nature of the heart of a hibernator has also become apparent from experiments in which attempts were made to induce artificial hypothermia in both hibernators and nonhibernators. When extreme hypothermia was forcibly induced by pentobarbital anesthesia combined with cooling of the whole body, cardiac contractility was maintained in hamsters (Miyazawa et al. 2008). This is in sharp contrast to nonhibernators, in which cardiac arrest is usually induced at a low temperature (Duker et al. 1983; Caprette and Senturia 1984; Johansson 1996). In fact, the same procedure for inducing artificial hypothermia in hamsters was lethal in rats (Miyazawa et al. 2008). In addition to the cold resistance, the heart of a hibernator is known to be resistant to various harmful stimuli. For instance, the heart of one of the hibernators, hedgehog dog, is hardly affected by manipulations that elicit atrial fibrillation (e.g., aconitine administration, high concentration of $CaCl_2$ administration, or ligation of the hepatic artery) (Johansson 1985, 1996).

3.5.2 Adaptive Changes in the Heart Prior to Hibernation

As mentioned above, the specific innate characteristics of the heart of hibernators would be important for enabling hibernation. It should be noted, however, that maintenance of cardiac function during hibernation does not totally depend on the innate ability of the heart. It is believed that adaptive changes that occur in response to a short photoperiod and cold ambient temperature are also essential for entering deep hibernation, as well as for keeping a hypothermic state and for recovery to a euthermic state. Therefore, numerous studies have been carried out to reveal remarkable differences between hibernating animals and their euthermic counterparts.

The most striking adaptive changes in the heart of hibernating animals are alterations in intracellular Ca^{2+} mobilization involving cardiac excitation-contraction coupling (Kondo and Shibata 1984; Lakatta and Guarnieri 1993). In general, intracellular Ca^{2+} for contraction of cardiac muscle is supplied by its entry into the cell through the L-type Ca^{2+} channel followed by Ca^{2+} release from the sarcoplasmic reticulum, a Ca^{2+} storage organelle (Kurihara 1994). In hibernating chipmunks, it has been demonstrated that activity of the L-type Ca^{2+} channel is suppressed and thereby entry of extracellular Ca^{2+} is limited (Kondo and Shibata 1984). Since excessive Ca^{2+} entry and its overload would damage cardiomyocytes through induction of apoptosis and/or necrosis, maintenance of Ca^{2+} homeostasis is essential for preventing profound arrhythmia and ventricular fibrillation (Lakatta and Guarnieri 1993). Thus, it can be considered that suppression of the L-type Ca^{2+} channel activity is an appropriate adaptive event for hibernating animals. Meanwhile, suppression of the channel activity may have a negative impact on cardiac contractility. To compensate for the reduced Ca^{2+} entry, release of Ca^{2+} from intracellular stores is enhanced during hibernation (Kondo and Shibata 1984). It is also important, in addition to the increased release, that reuptake of Ca^{2+} by the sarcoplasmic reticulum is enhanced (Belke et al. 1991). Collectively, suppression of channel activity in the plasma membrane with concomitant activation of store function enables efficacious Ca^{2+} cycling at a cold temperature.

3.5.3 Molecular Basis for the Adaptive Changes in the Heart of Hibernating Animals

As mentioned above, cardiac myocytes of hibernating mammals are characterized by reduced Ca^{2+} entry into the cell membrane (Alekseev et al. 1996; Yatani et al. 2004; Dibb et al. 2005) and a concomitant enhancement of Ca^{2+} release from and reuptake by the sarcoplasmic reticulum (Kondo and Shibata 1984; Belke et al. 1991; Wang et al. 2002). These adaptive changes would help in preventing excessive Ca^{2+} entry and its overload and in maintaining the resting levels of intracellular Ca^{2+} (Wang et al. 2002). The molecular basis of reduced Ca^{2+} entry into the cell membrane would not be due to reduced expression of the L-type Ca^{2+} channel protein but rather due to a decrease in channel activity by phosphorylation of the molecule (Kokoz et al. 2000) (Fig. 3.3).

As for the increased release of Ca^{2+} from intracellular stores, there has been a study demonstrating that the density of ryanodine receptors is increased in the sarcoplasmic reticulum, although the expression level of the receptors remains unchanged (Milner et al. 1991). The ryanodine receptor is the major Ca^{2+} release channel on the sarcoplasmic reticulum required for excitation-contraction coupling in cardiac muscle (Kurihara 1994). In addition, expression of sarco(endo) plasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a) is upregulated, and a negative regulator of SERCA2a, phospholamban (PLB), is downregulated during hibernation (Brauch et al. 2005). These changes enable a prompt removal of cytosolic Ca^{2+}, thereby ensuring diastolic filling (Fig. 3.3).

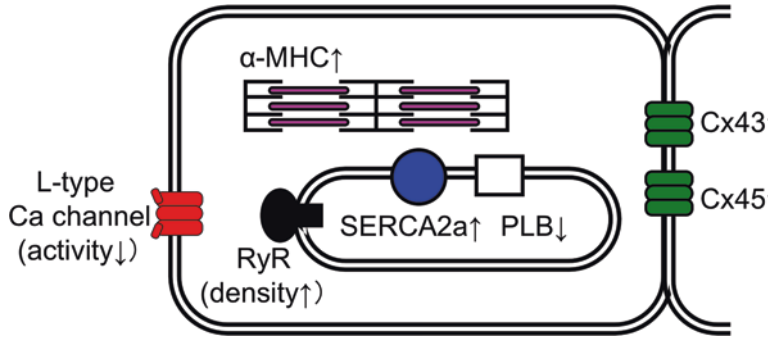

Fig. 3.3 Adaptive changes in molecules related to maintenance of intracellular Ca^{2+}, contractility or synchronous contraction in cardiac myocytes of hibernating animals. *RyR* ryanodine receptors, *SERCA2a* sarco(endo)plasmic reticulum Ca^{2+}-ATPase 2a, *PLB* phospholamban, *α-MHC* myosin heavy chain-α, *Cx* connexin

Moreover, the expression of functional proteins related to contractility (e.g., myosin heavy chain-α, ventricular myosin light chain, and the troponin C) and the expression of proteins involved in synchronous contraction (e.g., connexin43) have been shown to be upregulated or downregulated appropriately in hibernating animals (Saitongdee et al. 2000; Brauch et al. 2005; Fedorov et al. 2005). Importantly, the onset of these changes precedes the onset of hibernation (Kondo 1987; Saitongdee et al. 2000), indicating that these changes in gene expression and subsequent functional remodeling are preparatory processes for entering hibernation and are therefore indispensable for acquiring cold resistance (Fig. 3.3).

3.5.4 Autonomic Regulation of the Heart During Hibernation

The adaptive changes prior to hibernation would alone be insufficient to maintain cardiac pulsatility under an extremely hypothermic condition during hibernation, although these changes are undoubtedly indispensable. The operation of autonomic regulation for maintaining proper cardiac pulsatility during hibernation has been suggested by experiments focusing on artificially induced hypothermia in hamsters. By combining pentobarbital anesthesia with cooling of the animal, forced hypothermia that is comparable to that in hibernating animals can be successfully produced (Miyazawa et al. 2008). This procedure may reproduce a hypothermic condition without promoting possible autonomic functions that would usually be triggered in natural hibernation.

Even after sufficient exposure to an environment that is appropriate for induction of adaptive changes, hamsters show abnormal electrocardiograms (ECG) such as J wave and/or signs related to atrioventricular block when the hypothermic condition is forcibly induced (Miyazawa et al. 2008). The J wave, which is typically described in hypothermia in nonhibernating mammals (Brunson et al. 2005), is a wave located at the point of the end of the QRS complex and occupying the initial part of the ST segment (Gussak et al. 1995). The origin of the J wave during hypothermia has been attributed to injury current, delayed ventricular depolarization and early repolarization, tissue anoxia, and acidosis (Brunson et al. 2005). If the adaptive changes exclusively contribute to cold tolerance of the heart, heart pulsatility of well-adapted hamsters can be maintained appropriately not only during natural hibernation but also during a forcibly induced hypothermic condition. Therefore, the fact that the J wave as well as abnormal ECG signs related to conduction block are not observed in natural hibernation (Mertens et al. 2008; Miyazawa et al. 2008) can be rationally explained by the operation of regulatory mechanisms during natural hibernation to coordinate the cardiac

conducting system properly and to prevent cardiac impairment caused by hypothermia. The precise regulatory mechanisms have so far remained elusive.

3.6 Mechanism of Protection Against Cold Temperature

3.6.1 Cold-Shock Proteins-Associated Protection During Hibernation

Generally, the heart of mammalians cannot keep beating in a deep hypothermic condition (Ivanov 2000), suggesting that a cold temperature is harmful to the heart. In contrast, the heart of hibernating animals is capable of maintaining constant beating despite a decrease in body temperature to less than 10 °C during hibernation (Carey et al. 2003). Therefore, in addition to the adaptive changes prior to entering hibernation and the operation of autonomic regulatory mechanisms during hibernation, protection of cardiomyocytes against harmful effects of a cold temperature would be essential to maintain heart function under a condition of extreme hypothermia. In relation to the protective mechanism, recent studies have been focused on functional roles of cold-shock proteins, including cold-inducing RNA-binding protein (CIRP) and RNA-binding motif 3 (RBM3) (Zhu et al. 2016). It has been demonstrated that CIRP and RBM3 are induced by cold stress in cultured cells (Nishiyama et al. 1997; Gotic et al. 2016; Zhu et al. 2016). These proteins regulate gene expression at the level of translation (i.e., mRNA splicing, stability, and transport) and thus allow cells to respond rapidly to cold stress (Lleonart 2010; Zhu et al. 2016). Accumulating evidence indicates that CIRP and RBM3 play important roles in the protection of various types of cells against harmful effects of a cold temperature (Gualerzi et al. 2003; Saito et al. 2010).

The prominent action of cold-shock proteins, which was originally revealed in cells of nonhibernators, has been shown to function during hibernation. For example, it has been reported that RBM3 is increased in hibernating mammals such as black bears (Fedorov et al. 2009, 2011) and ground squirrels (Epperson et al. 2004; Williams et al. 2005) and plays an important role in neuroprotection (Tong et al. 2013; Peretti et al. 2015). Also, CIRP is expressed in response to a cold stress in the treefrog (Sugimoto and Jiang 2008). Accordingly, cold-shock proteins might help to protect organs including the heart against a harmful low temperature during hibernation.

3.6.2 Hibernation-Specific Alternative Splicing of the *CIRP* Gene

We recently reported the unique expression pattern of *CIRP* in the hearts of hibernating hamsters (Sano et al. 2015). In our study, RT-PCR analysis revealed that CIRP mRNA is constitutively expressed in the heart of a nonhibernating euthermic hamster with several different forms probably due to alternative splicing. The short product contained the complete open reading frame for full-length CIRP. On the other hand, the long product had inserted sequences containing a stop codon, suggesting production of a C-terminal deletion isoform of CIRP. The RNA-binding domain in the N-terminal region (Lleonart 2010) is conserved in the long isoform, indicating that the isoform possesses RNA-binding activity equal to that of full-length CIRP. However, the isoform lacks critical phosphorylation and methylation sites located in the C-terminal region, the phosphorylation and/or methylation of which is related to activation of CIRP (De Leeuw et al. 2007; Lleonart 2010). It is thus probable that the C-terminally truncated isoform plays a dominant-negative role over the full-length CIRP. In contrast to nonhibernating hamsters, only the short product is expressed in hibernating animals. It is therefore speculated that the dominant-negative regulation is important to mask the function of CIRP under a nonhibernating condition. The dominant-negative regulation combined with constitutively active transcription may permit rapid expression of CIRP function by switching the splicing pattern, leading to avoidance of hypothermic damage in the heart (Fig. 3.4).

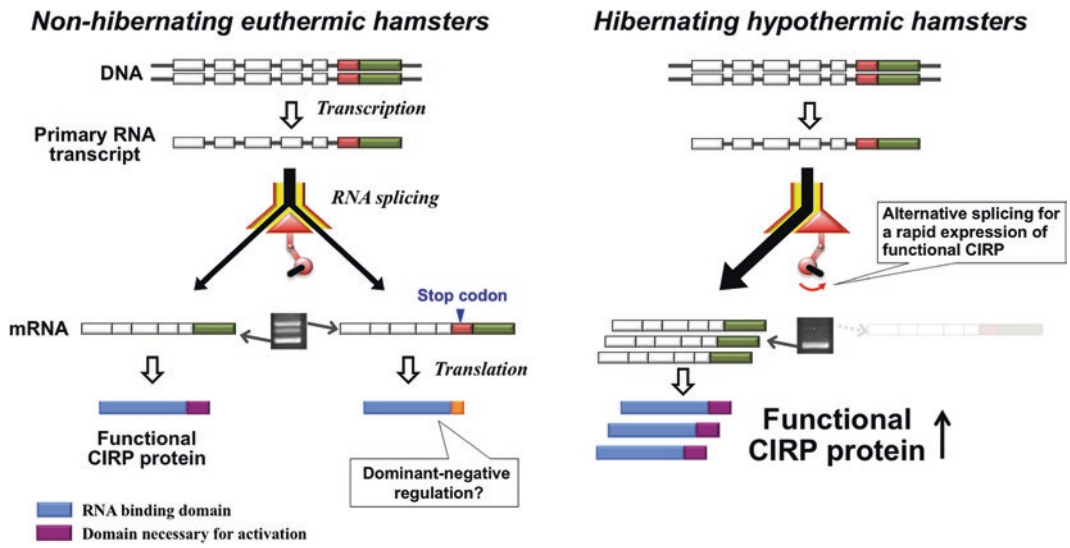

Fig. 3.4 Alternative splicing of cold-inducible RNA-binding protein (CIRP) gene in nonhibernating euthermic and hibernating hypothermic hamsters. (The figure was modified from our published article Sano et al. 2015)

It would be of interest to uncover the factors causing the shift in alternative splicing of CIRP. Recent evidence from cultured cells suggests that a mild cold temperature (32 °C) is a possible trigger for splicing regulation of the CIRP gene, since mild cold exposure increases the expression of CIRP mRNA without affecting its pre-mRNA levels (Gotic et al. 2016). This assumption can be applicable to the shift in alternative splicing of CIRP under the condition of natural hibernation, since animals go through a gradual decrease in body temperature, and they would maintain mild hypothermia, about 25–30 °C, for several hours (Horwitz et al. 2013). Taken together, it is reasonable to consider that mild hypothermia during the induction period of hibernation might induce hibernation-specific alternative splicing of CIRP in the hamster heart.

3.7 Induction of Artificial Hypothermia in Nonhibernating Animals

3.7.1 Significance of Therapeutic Hypothermia

Hypothermia results in a reduction of cellular metabolic rate and oxygen consumption, indicating that it may have therapeutic efficacy (Hale and Kloner 2011; Tissier et al. 2012). Mild hypothermia, 32–35 °C, is very potent for reducing myocardial infarct size in some experimental animal models such as rabbits, dogs, sheep, pigs, and rats (Tissier et al. 2012). In addition, induced hypothermia has been shown to reduce the risk of cerebral ischemic damage both in animal studies and in humans, who have been resuscitated following cardiac arrest (Galvin et al. 2015). Thus, it is important to devise a method by which hypothermia can be induced safely and simply in nonhibernating mammals including humans.

Therapeutic hypothermia is generally induced by a combination of anesthesia with cooling in the patient (Galvin et al. 2015). In addition, safe and simple pharmacological approaches to achieve therapeutic hypothermia have been investigated. For instance, hydrogen sulfide can induce a state of hypothermia in mice by inhibiting cytochrome oxidase, which decreases their metabolic rate and core body temperature (Guo et al. 2012). Administration of capsaicin also reduces body temperature by about 2–3 °C (Jakab et al. 2005; Swanson et al. 2005; Jones et al. 2009; Dow et al. 2014) since capsaicin is an agonist of TRPV1, which can detect a painful hot temperature (>42 °C) (Montell and Caterina 2007) and would be recognized as heat exposure, leading to reduction of body temperature mediated by the thermoregulatory center. It should be

noted, however, that the target temperature is generally about 30 °C, which is categorized as mild hypothermia, and that it is difficult to induce hibernation-like extreme hypothermia even by these methods.

3.7.2 Induction of Hypothermia by Activation of Central Adenosine A1-Receptor

One of the profound problems that occur during induction of artificial hypothermia is heart dysfunction such as ventricular fibrillation and cardiac arrest. Even in hibernators such as hamsters, abnormal ECG is recorded during nonhibernation artificial hypothermia induced by pentobarbital anesthesia and cooling (Miyazawa et al. 2008). To devise a safe method for induction of hypothermia, elucidation of the mechanisms for tolerance to cold stress during hibernation would provide valuable information. Central adenosine A1-receptor-mediated signals play a role in the induction and maintenance of hibernation (Tamura et al. 2005; Jinka et al. 2011; Iliff and Swoap 2012). The predominant role of adenosine A1-receptor-mediated signals leads to the idea that activation of adenosine A1-receptors would induce hypothermia in both hibernating and nonhibernating mammals. In accordance with this, central administration of an adenosine A1-receptor agonist and subsequent cooling induces extreme hypothermia in hamsters (Miyazawa et al. 2008) and rats (Tupone et al. 2013; Shimaoka et al. 2018) without accompanying atrioventricular block or abnormal ECG. These findings suggest that central adenosine A1-receptor-mediated signals would provide an appropriate condition for maintaining normal sinus rhythm during extreme hypothermia.

3.8 Conclusion

Entering hibernation in mammals confers resistance to various harmful events such as low body temperature, severe ischemia, bacterial infection, irradiation, and muscle disuse. Therefore, hibernation mechanisms are considered to be a potential therapeutic target for the treatment of several diseases. Although the application of this unique phenomenon to medical fields has been strongly desired, a poor understanding of the mechanisms limits the progress toward developing novel therapeutic strategies. A large number of previous experiments focused on adaptive changes in the heart prior to hibernation. It is clear that adaptive changes are involved in the beneficial properties of hibernating animals. However, it remains unclear whether these changes are solely responsible for the establishment of a hibernating condition. For instance, it is uncertain whether the changes at the molecular level (see Fig. 3.3) are sufficient for maintaining cardiac pulsatility under an extremely hypothermic condition. On the other hand, artificially induced hypothermia may provide a valuable tool to answer the question. The method for inducing hypothermia forcibly in hamsters allows reproduction of a hypothermic condition in the absence of possible hibernation-specific reactions. Unlike hypothermia in natural hibernation, the forced induction of hypothermia causes irreversible injury of the myocardium (Miyazawa et al. 2008). Comparison of the heart in forced hypothermia with that during hibernation would be valuable for identifying critical factors related to cold resistance of the heart. Thus, it is expected that further studies using artificial hypothermia may provide a breakthrough in understanding the hibernation mechanisms.

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4 Freezing Tolerance of Plant Cells: From the Aspect of Plasma Membrane and Microdomain

Daisuke Takahashi, Matsuo Uemura, and Yukio Kawamura

Abstract

Freezing stress is accompanied by a state change from water to ice and has multiple facets causing dehydration; consequently, hyperosmotic and mechanical stresses coupled with unfavorable chilling stress act in a parallel way. Freezing tolerance varies widely among plant species, and, for example, most temperate plants can overcome deleterious effects caused by freezing temperatures in winter. Destabilization and dysfunction of the plasma membrane are tightly linked to freezing injury of plant cells. Plant freezing tolerance increases upon exposure to nonfreezing low temperatures (cold acclimation). Recent studies have unveiled pleiotropic responses of plasma membrane lipids and proteins to cold acclimation. In addition, advanced techniques have given new insights into plasma membrane structural non-homogeneity, namely, microdomains. This chapter describes physiological implications of plasma membrane responses enhancing freezing tolerance during cold acclimation, with a focus on microdomains.

Keywords

Plant · Cold acclimation · Plasma membrane · Microdomain · Freezing tolerance · Proteome

Abbreviations

ACBP	Acyl-coenzyme A-binding protein
ASG	Acylated sterylglycoside
BCB	Blue-copper-binding protein
BI	Bax inhibitor
BRI	Brassinosteroid insensitive
CBF	C-repeat-binding factor
CPK	Calcium-dependent protein kinase
Cryo-SEM	Cryo-scanning electron microscopy
DRM	Detergent-resistant membrane
DRP	Dynamin-related protein
FLA	Fasciclin-like arabinogalactan protein
FLOT	Flotillin
FS	Free sterol
GH17	O-glycosyl hydrolase family 17

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GIPC	Glycosyl inositol phosphoryl ceramide
GPDL	Glycerophosphoryl diester phosphodiesterase-like protein
GPI	Glycosylphosphatidylinositol
H_{II}	Hexagonal II
HIR	Hypersensitive-induced reaction
LCB	Long-chain base
LCBK	LCB kinase
LTP	Lipid transfer protein
PDCB	Plasmodesmata callose-binding protein
PHS-P	4-Hydroxy-sphinganine-phosphate
PLD	Phospholipase D
PTM	Posttranslational modification
SG	Sterylglycoside
SGT	Sterol glycosyltransferase
SLAH	Slow anion channel 1 homolog
SLD	Sphingolipid Δ8 LCB desaturase
SYP	Syntaxin of plants
SYT	Synaptotagmin

4.1 Introduction

As immovable organisms, plants must continually monitor ambient conditions and properly respond to environmental changes. In spite of this limitation, plants have adapted to extreme environments ranging from aquatic habitats to alpine areas and constitute one of the most successful groups of organisms worldwide. In various environments, plants suffer from different abiotic stresses that homogenously, extensively, and species-nonspecifically influence plant growth and survival. Abiotic stresses include unfavorable temperatures, water unavailability, high salinity, inadequate light, and physical pressures. Among these stresses, freezing stress has the most critical effect on plant survival. Freezing stress is accompanied by a state change from water to ice and has multiple facets causing dehydration; consequently, hyperosmotic and mechanical stresses coupled with unfavorable chilling stress act in a parallel way (Steponkus 1984). While most temperate plants can overcome deleterious effects caused by freezing temperatures in winter, tropical and some temperate species cannot withstand such temperatures. Freezing tolerance thus varies widely among plant species and sometimes even within natural accessions of a single species.

One obvious question is what the key factor is for understanding freezing tolerance in plants. The most crucial component of plant cells under freezing conditions is the plasma membrane (Steponkus 1984). The disruption of the plasma membrane, which delineates extra- and intracellular environments, leads directly to cell death. Plasma membrane stability and flexibility are therefore deduced to be directly related to plant survival under freezing temperatures (Steponkus 1984). In addition, freezing stress is multifactorial: cold temperatures disrupt enzymatic activities and the physicochemical behavior of the plasma membrane, while extracellular freezing induces water migration from within the cell to extracellular space, increases osmotic concentration in unfrozen water, and puts mechanical pressure on the plant cell surface. Taken together, freezing stress is accompanied by cold, dehydration, osmotic, and mechanical stresses, all of which are more or less associated with plasma membrane function (Takahashi et al. 2013c). The plasma membrane is thus a key factor for overcoming complex freezing injury.

Because freezing tolerance is enhanced by cold acclimation using nonfreezing low temperatures such as 4 °C, the mechanisms of freezing tolerance have traditionally been studied by comparing plant samples before and after cold acclimation. In addition, comparisons between strongly and weakly freezing-tolerant plants, such as rye (*Secale cereale*) and oat (*Avena sativa*), have yielded a better understanding of plant freezing tolerance (Uemura and Yoshida 1984; Uemura and Steponkus 1989; Webb et al. 1994; Takahashi et al. 2013a). Recent advancements in proteomic and lipidomic technologies and the development of the lipid raft model, a new modification of the fluid mosaic model, have provided important information on complex changes of the plasma membrane caused by physiological shifts under stressed conditions. Genetic and physiological approaches using model plants have also unveiled sophisticated and subtle strategies used by plants to adapt to severe freezing.

In this review, general features of cold acclimation and freezing tolerance in plants are first

summarized. After describing advances in plasma membrane studies, various aspects raised by these studies and future perspectives in plant low-temperature biology are then discussed.

4.2 General Features of Cold Acclimation and Freezing Tolerance

Freezing stress is accompanied by several stress factors, all of which must be overcome by plants. Plant cells will otherwise be disrupted, leading in turn to death of the cell and eventually the individual organism. Detailed mechanisms of cold acclimation and freezing tolerance as survival strategies against severe freezing are discussed in this section.

4.2.1 Cold Acclimation as a Process Toward Adaptation to Freezing

To withstand severe freezing stress, temperate plants have developed a set of adaptation mechanisms referred to as cold acclimation. Cold acclimation is principally achieved via nonfreezing low temperatures and short-day conditions. The maximum freezing tolerance and optimal duration of cold acclimation vary with plant species. For example, oat and rye achieve their maximum freezing tolerances under cold acclimation treatment for 4 weeks, but their tolerances are different (−10 °C for winter oat and −15 °C for winter rye) (Webb et al. 1994). The model plant *Arabidopsis* also has the capacity for cold acclimation. The maximum freezing tolerance of *Arabidopsis*, −10 °C, is attained by cold acclimation treatment for 7 days (Uemura et al. 1995). Among natural accessions, however, the maximum freezing tolerance varies considerably, ranging from −8 to −14 °C (Hannah et al. 2006).

During cold acclimation, solutes, including sugars, amino acids, and specific proteins (e.g., dehydrin), accumulate to prevent membranes from undergoing freezing-induced denaturation and disruption (Koster and Lynch 1992; Welin et al. 1994; Danyluk et al. 1998; Wanner and Junttila 1999; Kosová et al. 2008). This process is regulated by the expression of specific genes such as C-repeat-binding factors (CBFs), which quickly increase in the first step of cold acclimation (Thomashow 1998, 1999). For instance, rye, a monocot plant, accumulates a variety of solutes, including proline, soluble sugars, and glycine betaine, during cold acclimation (Koster and Lynch 1992), and repartitions fructans and simple sugars within lower and upper crown tissues during freezing at −3 °C (Livingston et al. 2006). In *Arabidopsis*, several sugars, such as glucose, fructose, and sucrose, clearly increase during cold acclimation and decrease rapidly during de-acclimation (Zuther et al. 2015). Contents of these sugars as well as transcript abundances of *CBF1* and *CBF2* under cold acclimation have been found to be correlated with freezing tolerance in each natural accession of *Arabidopsis* (Zuther et al. 2015).

Remodeling of cell wall composition and structure has also been observed during the cold acclimation process in several species. Studies based on cryo-scanning electron microscopy (Cryo-SEM) have provided information on the cell wall as a barrier against extracellular freezing (Pearce 1988; Yamada et al. 2002). Changes in cell wall components such as crude cell wall, pectin, hemicellulose, and lignin are actually induced by cold acclimation treatment (Zabotin et al. 1998; Kubacka-Zębalska and Kacperska 1999; Solecka et al. 2008; Amid et al. 2012; Domon et al. 2013; Livingston et al. 2013; Baldwin et al. 2014; Ji et al. 2015). In winter oat, concentrations of apoplastic fructan, glucose, fructose, and sucrose increase during cold acclimation and sub-zero temperature acclimation immediately after cold acclimation (Livingston and Henson 1998). This response may also contribute to the prevention of ice crystal growth and propagation during freezing.

4.2.2 The Plasma Membrane as a Primary Site of Freezing Injury

In addition to the effects of chilling stress induced by low temperatures, freezing stress caused by sub-zero temperatures (<0 °C) has diverse impacts on plant cell survival (Levitt 1980; Steponkus 1984). For example, exposure to freezing temperatures is attended by a risk of an intracellular state change from water to ice, a lethal event. In plant cells that survive under freezing, this intracellular freezing does not generally take place, and ice nucleation occurs preferentially in extracellular space. Nevertheless, a difference in chemical potential is established between the extracellular ice and the intracellular solution, consequently leading to cellular dehydration. Plants must therefore overcome extracellular freezing-induced cellular dehydration. Because ice crystals exclude solutes, this extracellular ice formation additionally leads to increased concentrations of solutes in unfrozen portions of extracellular and/or intracellular solutions. Greatly concentrated solutes may cause osmotic (and/or salinity) stress in plant cells. Furthermore, physical pressure from enlarged extracellular ice crystals is deleterious to plant cells (Levitt 1980; Steponkus 1984). Freezing stress therefore has multiple aspects, including cold, dehydration, osmotic, and mechanical stresses (Fig. 4.1).

In any of the freezing-induced stresses, the plasma membrane is the most important factor determining plant cell survival. In particular, the plasma membrane acts as a barrier against invasion of extracellular ice into intracellular space during extracellular freezing-induced dehydration and mechanical stress. Intracellular freezing would otherwise occur and lead to cell death. Furthermore, the plasma membrane defines cell shape, surface area regulation, and trafficking between intracellular and extracellular spaces. Plasma membrane behavior and function may be very important for dealing with freezing-induced dehydration and hyperosmotic stresses in plant cells. Extended dehydration and osmotic stresses can prevent proper functioning of the plasma membrane through their harmful effects on physicochemical characteristics of plasma membrane components (Steponkus 1984). In addition, dehydration results in interbilayer fusion between the plasma membrane and other intracellular membranes, leading to injuries referred to as fracture-jump lesions and lamellar-to-hexagonal-II (H_{II}) phase transitions (Steponkus 1984; Gordon-Kamm and Steponkus 1984a; Webb and Steponkus 1993; Webb et al. 1993, 1994). When endomembranes are in close apposition to the plasma membrane, fracture-jump lesions or H_{II} phase transitions are thought to arise by structuration between lipid bilayers of both membranes. H_{II} phase transitions, in particular, are accompanied by the formation of a long cylinder-like structure with circularly arranged polar head groups of the membrane. These freezing-induced membrane structures are considered to trigger demixing and lateral segregation of principal membrane components such as lipids and proteins, thereby disrupting normal functions of the plasma membrane.

4.2.3 Significance of the Plasma Membrane in the Enhanced Freezing Tolerance After Cold Acclimation

Most of the cold acclimation-related events mentioned above are strongly associated with the enhancement of plasma membrane stability. For example, accumulations of sugars and dehydrins are important events that prevent interbilayer fusion of the plasma membrane with other endomembranes (Gordon-Kamm and Steponkus 1984a, b; Steponkus et al. 1988; Uemura and Steponkus 1989; Webb and Steponkus 1993; Webb et al. 1993, 1994, 1995; Danyluk et al. 1998; Ashraf and Foolad 2007; Eriksson et al. 2011; Rahman et al. 2013; Thalhammer et al. 2014). These solutes can interact with the plasma membrane under freezing-induced dehydration conditions and maintain a certain distance between the plasma membrane and intracellular organelle membranes. Alternatively, sugars and amino acids such as prolines contribute to

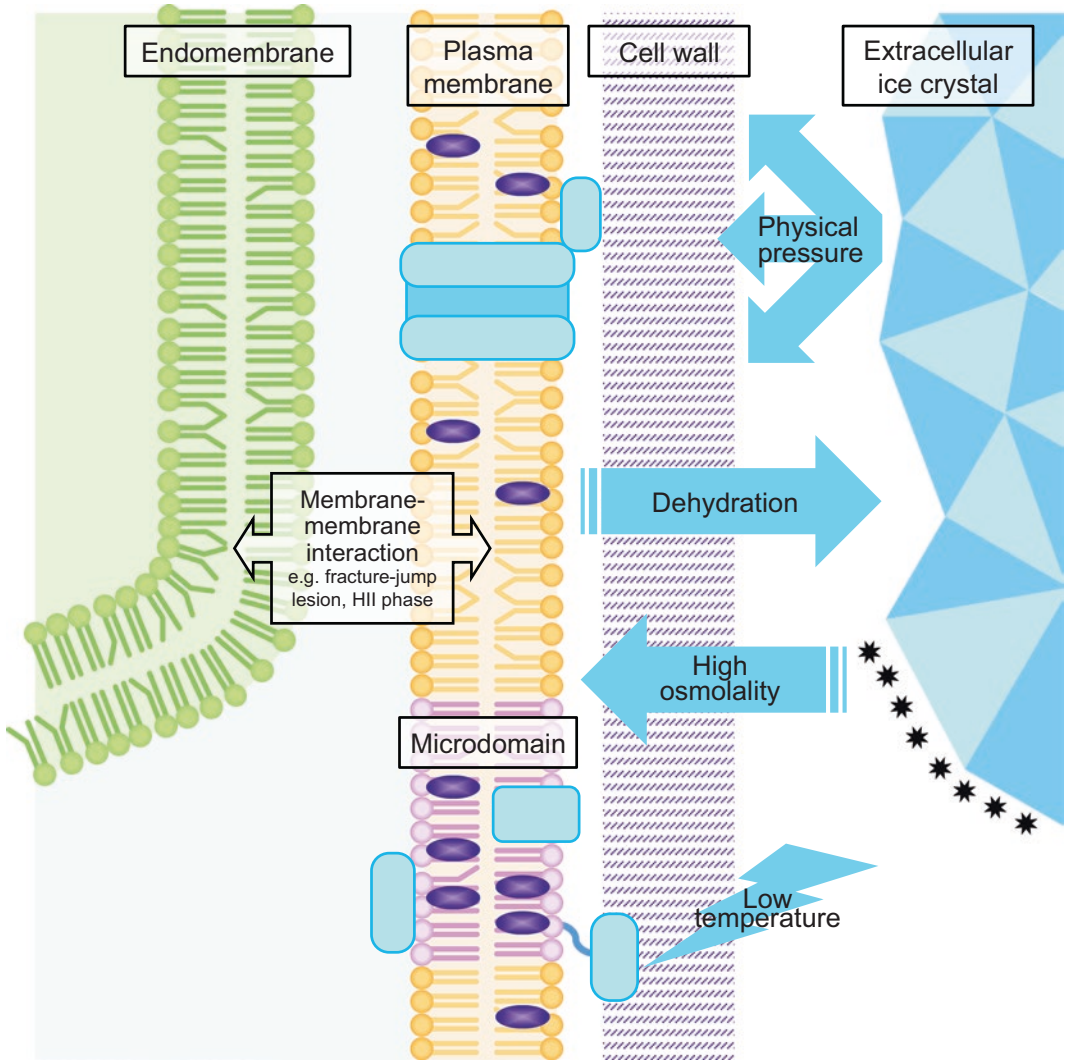

Fig. 4.1 Schematic illustration of freezing injury. Low temperatures influence the functions of plasma membrane-associated proteins. Extracellular ice formation induces water absorption from intracellular space. Expanded ice crystals impose physical pressure on the plasma membrane surface. Eventually, the plasma membrane can be fused with closely positioned endomembranes. Condensed solutes in extracellular unfrozen water lead to osmotic stress and dysfunction of the plasma membrane

scavenging of free radicals, osmotic adjustment, and buffering of cellular redox potentials. Although the extracellular matrix is not critical to plant survival under freezing conditions, since protoplast has a substantial freezing tolerance (Gordon-Kamm and Steponkus 1984a, b; Steponkus et al. 1988; Uemura and Steponkus 1989), extracellular responses such as modification of cell walls and accumulation of apoplastic solutes may help prevent penetration of ice from extracellular space through the plasma membrane into the cell (Yamada et al. 2002). On the basis of all this evidence, the plasma membrane is inferred to be the principal site affected by changes in intracellular and extracellular compartments in response to cold acclimation. In other words, protection of the plasma membrane takes highest priority under freezing conditions, and the stability of the plasma membrane determines plant freezing tolerance.

4.3 Changes of the Plasma Membrane in Response to Cold Acclimation

As mentioned above, the plasma membrane is the most crucial component for plant survival under freezing temperatures. The plasma membrane is composed of lipids and proteins, both of which should be affected both quantitatively and qualitatively to enhance freezing tolerance. In other words, cold acclimation alters lipid and protein compositions and functions to enhance plasma membrane integrity and stability under freezing temperatures. Modifications that take place after translation, such as glycosylation, may also be important factors that change plasma membrane properties. In addition, the structural model of the plasma membrane has been updated as the result of new findings. Along with technological developments, changes of the plasma membrane in response to cold acclimation have thus been studied from various angles.

4.3.1 Components and Structure of the Plasma Membrane

The two major components of the plasma membrane are lipids and proteins. Plasma membrane lipids are mainly composed of phospholipids, sphingolipids, and sterols. Each lipid class contains a wide range of members. For instance, more than 300 different types of sphingolipids have been estimated in various kinds of organisms (Hannun and Luberto 2000). Basically, the predominant structure of the plasma membrane is the lipid bilayer. Most plasma membrane lipids have both a hydrophilic head and a hydrophobic tail in one molecule; the exception is sterol lipids, which contain a very hydrophobic ring structure and, in some cases, sugar and acyl moieties. Head groups are always oriented to the outside of the bilayer, while tails face each other toward the center of the two-layered sheets. Head groups have specific structures and diverse properties. Because it can determine physical and chemical properties of the plasma membrane surface, the molecular assembly of head groups plays an important role in overall cell functionalities and characteristics.

In addition to lipids, the plasma membrane contains a remarkably diverse population of proteins. Tanz et al. (2013) confirmed at least 4000 proteins in *Arabidopsis* as plasma membrane-localized proteins by GFP tagging and mass spectrometry. There are several categories of plasma membrane proteins. For example, some plasma membrane proteins are integrated into the lipid bilayer, while others are weakly attached to the surface. These proteins play important roles in transportation of ions and small molecules, signal transduction, synthesis of extracellular components, secretions of proteins and other molecules, and intracellular and/or intercellular vesicle trafficking.

Some of these membrane proteins are dynamically modified after translation (i.e., posttranslational modification or PTM). More than 300 different PTM mechanisms have been reported (Zhao and Jensen 2009; Kline-Jonakin et al. 2011), with the ones most highly emphasized in plasma membrane studies being phosphorylation, acetylation, glycosylation, and oxidation. These PTMs possibly regulate protein activity, stability, localization, and interactions with other molecules (Yadeta et al. 2013). Furthermore, lipid-anchored proteins, such as glycosylphosphatidylinositol (GPI)-anchored, myristoylated, palmitoylated, and prenylated proteins, are covalently bound to plasma membrane lipids. Some PTMs such as glycosylation and lipid modification can be further diversified by various bonding partners.

Modern biochemical and cell biological techniques have yielded new insights into plasma membrane structure. In the past, the structure of the plasma membrane was described in terms of the fluid mosaic model proposed by Singer and Nicolson (1972). In the fluid mosaic model, components of the plasma membrane such as proteins and lipids are considered to be laterally diffused at random. This model is still widely supported by many studies based on animal, plant, yeast, and artificial liposome systems. More recently, however, several studies have uncovered the existence of microdomains formed

in the plasma membrane by lateral segregation of specific components such as sphingolipids, sterols, and certain proteins (Simons and Ikonen 1997). These microdomains are of various sizes (Kusumi et al. 2005) and have been implicated in many plant cell physiological processes, such as pollen tube tip growth, intercellular virus movement, responses to iron deficiency, and regulation of various membrane proteins including brassinosteroid insensitive1 (BRI1), hypersensitive-induced reaction (HIR) proteins, Rac/ROP small GTPase (Rac1), and respiratory burst oxidase homologs (Raffaele et al. 2009; Liu et al. 2009; Hao et al. 2014; Wang et al. 2015; Nagano et al. 2016; Gutierrez-Carbonell et al. 2016; Lv et al. 2017).

Along with the current consensus that microdomain components are mainly composed of hydrophobic molecules, microdomains are thought to be obtainable as nonionic detergent-resistant membrane (DRM) fractions at chilling temperatures. This latter postulation, however, is still debatable. On the one hand, DRMs do not always reflect the structure and nature of membrane microdomains (Tanner et al. 2011; Malinsky et al. 2013). On the other hand, remorin, a major DRM protein, is becoming recognized as a microdomain marker because it organizes patch-like structures in the plasma membrane that can be disrupted by treatment with a sterol chelator (methyl-β-cyclodextrin) (Kierszniowska et al. 2009; Raffaele et al. 2009). At a minimum, however, analysis of DRM fractions on a large scale seems to be a suitable first step to uncover the association between specific nanostructures of the plasma membrane and various physiological processes.

4.3.2 Lipidomic Changes of the Plasma Membrane During Cold Acclimation

As mentioned above, lipids are a principal component of the plasma membrane. The molecular composition of plasma membrane lipids and their molecular changes during cold acclimation have been investigated in many studies of various plant species. In early studies, both compositional analysis of lipids and comparative analysis before and after cold acclimation were performed using plasma membrane fractions (Steponkus 1984; Yoshida and Uemura 1984; Uemura and Yoshida 1984; Ishikawa and Yoshida 1985; Palta et al. 1993; Uemura and Steponkus 1994; Uemura et al. 1995). Following the incorporation of the microdomain or lipid raft model into the fluid mosaic model of the plasma membrane, several reports emerged of changes to microdomain lipid composition during cold acclimation (Örvar et al. 2000; Minami et al. 2009; Degenkolbe et al. 2012; Takahashi et al. 2016a). More recently, improvements to lipidomic analysis techniques (mainly driven by advances in mass spectrometric methods) have allowed more in-depth analysis of membrane lipids (Degenkolbe et al. 2012; Vu et al. 2014; Tarazona et al. 2015; Takahashi et al. 2016a).

4.3.2.1 Phospholipids

Early studies dealing with plasma membrane lipids and cold acclimation focused on lipid class composition and the degree of unsaturation of plasma membrane lipids. For example, Uemura and Steponkus (1994) initially revealed that proportions of phospholipids and of unsaturated ones (mainly phosphatidylcholine and phosphatidylethanolamine) significantly increased in the plasma membrane over 4 weeks of cold acclimation in both rye and oat. These trends were also observed in *Arabidopsis* leaves (Uemura et al. 1995). Increases in the proportion of highly hydrated lipids such as phospholipids and the degree of unsaturation decrease hydrophobicity at the membrane surface and increase membrane fluidity, respectively. Even if plant cells are extremely dehydrated by freezing, the plasma membrane can consequently be kept spatially separate from intracellular membranes because of water molecules bound to the surface of the plasma membrane. The most significant impact of these changes is thus considered to be a decrease in the freezing-induced formation of the H_{II} phase; this is because the lamellar-to-H_{II} transition is an interbilayer event that occurs at

the site of closely apposed membranes derived from freezing-induced dehydration.

In DRM fractions, however, the propensity toward cold acclimation-induced changes observed in the plasma membrane is not applicable (Minami et al. 2009; Takahashi et al. 2016a). For example, phospholipid proportions in DRMs are slightly decreased by cold acclimation treatment in *Arabidopsis* (Minami et al. 2009) and are relatively steady during cold acclimation in rye (Takahashi et al. 2016a). These results indicate that microdomains have different properties than other plasma membrane areas and function as a scaffold for various physiological responses during cold acclimation.

4.3.2.2 Sphingolipids

Sphingolipids, which are a putative major component of plant microdomains, are also affected by cold acclimation treatment. Glucocerebrosides, one of the major sphingolipid classes, generally decrease in both monocot and dicot plants to prevent undesirable phase transitions under freezing temperatures (Lynch and Steponkus 1987; Uemura and Steponkus 1994; Uemura et al. 1995). In DRM fractions, however, similar to phospholipids, few changes take place in glucocerebroside proportions during cold acclimation (Minami et al. 2009; Takahashi et al. 2016a); this suggests that the structure of the sphingolipid-enriched microdomain is preserved, with its function consequently partially maintained and/or fulfilled even during cold acclimation when the total sphingolipid proportion in the plasma membrane decreases. Glycosyl inositol phosphoryl ceramide (GIPC) is another predominant sphingolipid class (Markham et al. 2006) in addition to glucocerebrosides but still remains poorly characterized (Buré et al. 2014). No information therefore exists about the changing patterns of GIPC and its significance in the plasma membrane and microdomains during cold acclimation.

Long-chain base (LCB), a major component of sphingolipid molecules, plays an important role in plant cold response. Cold treatment induces transient formation of 4-hydroxy-sphinganine-phosphate (PHS-P), a phosphorylated LCB, via enhanced activity of LCB kinase 2 (LCBK2) (Dutilleul et al. 2012). LCBK1 also potentially determines plant freezing tolerance through regulation of reactive oxygen species homeostasis (Huang et al. 2017). Sphingolipid Δ8 LCB desaturase 1 (SLD1) is associated with response to cold temperatures together with Bax inhibitor-1 (BI-1) (Nagano et al. 2014). Interestingly, BI-1 determines the abundance of representative DRM proteins, flotillin homolog (FLOT), and hypersensitive-induced reaction protein 3 (HIR3) in *Arabidopsis* DRM fractions (Ishikawa et al. 2015). Recent studies have also demonstrated that SLD is a protein associated with chilling injury of chloroplasts in tomato (Zhou et al. 2016). Although many studies have revealed that the metabolism of sphingolipid and its derivatives is important for cold response, the involvement of the sphingolipid-enriched microdomain itself in freezing tolerance and cold acclimation has not yet been fully characterized.

4.3.2.3 Sterols

Sterols are another component of the plasma membrane and microdomains. Previous studies of *Arabidopsis*, oat, and rye (Uemura and Steponkus 1994; Uemura et al. 1995) have noted a decrease in glycosylated sterols such as sterylglycosides (SGs) and acylated sterylglycosides (ASGs) and an increase in free sterols (FSs). Because of the hydrophobicity of sterol and acyl moieties in the ASG molecule, lowering of the proportion of ASGs would be expected to help prevent dehydration-induced formation of the H_{II} phase under freezing-induced dehydration conditions (Webb et al. 1995).

Sterol compositions of DRM fractions of different plant species show specific responses to cold acclimation (Minami et al. 2009; Takahashi et al. 2016a), which suggests that sterols embedded in the lipid bilayer of microdomains have physiological significance related to modulation of activities of microdomain-recruited proteins in association with freezing tolerance. Interestingly, transfer of the sterol glycosyltransferase (SGT) gene isolated from *Withania somnifera* to *Arabidopsis* confers salt, heat, and cold stress tolerance (Mishra et al. 2013, 2015). Analysis of knockout lines of the

Arabidopsis SGT gene, *TTG15/UGT80B1*, has revealed that cold-acclimated plants, but not non-acclimated ones, have a decreased survival rate after freeze-thawing (Mishra et al. 2015). A decrease in ASGs and an increase in FSs may therefore improve membrane stability under freezing conditions; at the same time, these glycosylated sterols are important for freezing tolerance acquisition and potentially contribute to microdomain functions such as assisting cellulose production during cold acclimation (Endler and Persson 2011).

4.3.3 Proteomic Changes of the Plasma Membrane During Cold Acclimation

Changes in the proteomic profile of the plasma membrane during cold acclimation have been characterized in *Arabidopsis*, orchard grass, oat, and rye. Two pioneer studies (Uemura and Yoshida 1984; Yoshida and Uemura 1984) reported compositional changes of plasma membrane proteins during cold acclimation by SDS-PAGE analysis. Mass spectrometric approaches have contributed to the identification of plasma membrane proteins isolated from *Arabidopsis* (Kawamura and Uemura 2003; Minami et al. 2009). Shotgun proteomic analysis has allowed high-throughput identification and quantification of plasma membrane proteins isolated from several plant species (Li et al. 2012a; Takahashi et al. 2012, 2013a, 2016b). In addition, advances in proteomic technologies, including increased mass spectrometer sensitivity, have allowed the detection of less abundant proteins in plant cells. DRM proteins are generally extracted in small amounts (only roughly 10–20% of total plasma membrane proteins in tobacco, *Arabidopsis*, oat, and rye) (Mongrand et al. 2004; Minami et al. 2009; Takahashi et al. 2012). Several early studies relying on classical gel-based proteomics have characterized the compositions of DRM proteins in various plant species (Mongrand et al. 2004; Borner et al. 2005; Morel et al. 2006; Laloi et al. 2006; Lefebvre et al. 2007; Krügel et al. 2008; Minami et al. 2009; Fujiwara et al. 2009). Taken together, these results suggest that plant DRM fractions typically accumulate various functional proteins, such as P-type ATPase, aquaporin, remorin, tubulin, leucine-rich repeat receptor like kinase, NADPH oxidase, hypersensitive-induced reaction proteins (Band 7 family proteins), and glucan synthase (reviewed in Takahashi et al. (2013b)). Recent studies using Orbitrap technologies have revealed an even greater diversity of plasma membrane proteins accumulating in DRM fractions; these proteins include several GPI-anchored proteins, arabinogalactan proteins, and heat shock proteins (Kierszniowska et al. 2009; Takahashi et al. 2012, 2013a, 2016b; Gutierrez-Carbonell et al. 2016).

Some DRM proteins have been characterized with a focus on the effects of sterol depletion on their distribution into DRM fractions. As studied by Kierszniowska et al. (2009) and reviewed by Tapken and Murphy (2015), proteomes of typical DRM proteins, such as fasciclin-like arabinogalactan proteins, FLOTs, and glycosyl hydrolases, show responses to sterol depletion treatments, consistent with the concept of microdomains. Not all DRM-enriched proteins, however, are affected by sterol depletion treatments, which suggest that various kinds of microdomains exist in the plasma membrane. On the other hand, some DRM proteins have been identified as actual microdomain components of the plasma membrane in plant cells. Remorin, the best-characterized DRM protein, is recognized as a lipid raft marker because it localizes as a patch-like structure on the plasma membrane (Raffaele et al. 2009; Furt et al. 2010; Jarsch et al. 2014; Bozkurt et al. 2014; Konrad et al. 2014; Frescatada-Rosa et al. 2014). Slow anion channel 1 homolog 3 (SLAH3) co-localizes with calcium-dependent protein kinase 21 (CPK21) and remorin 1.3 on the plasma membrane (Demir et al. 2013). FLOTs, KAT1 (K^+ channel), plasma membrane intrinsic protein2;1 (PIP2;1), and syntaxin of plants 21 (SYP21) also exhibit patch-like distributions on the plasma membrane (Bhat et al. 2005; Li et al. 2011, 2012b; Jarsch et al. 2014). The auxin transporter PIN1 interacts with ABCB19 in the microdomain compartment on

the plasma membrane, and sphingolipid and sterol biosynthesis is correlated with *abcb9* phenotypes, which suggests that sphingolipid- and sterol-enriched microdomains have significant roles in fundamental physiological processes (Titapiwatanakun et al. 2009; Yang et al. 2013). Consequently, lipid remodeling in the plasma membrane during cold acclimation is deduced to be closely related to microdomain dynamics and lateral distribution of plasma membrane proteins.

Taking into consideration all the results of proteomic studies of cold acclimation, the following events can be hypothesized to occur during cold acclimation in DRM proteins: (1) an increase in P-type H^+-ATPases, (2) disassembly of cytoskeletal components (such as tubulin) in the juxtamembrane region, (3) rearrangement of vesicle trafficking proteins, and (4) accumulation of membrane protection proteins on the plasma membrane surface. Furthermore, the DRM proteins synaptotagmin 1 (SYT1), dynamin-related protein 1E (DRP1E), and phospholipase D (PLD), all of which increase during cold acclimation, have been functionally studied in detail.

SYT1 has been identified as a cold acclimation-induced protein in DRM fractions (Kawamura and Uemura 2003; Minami et al. 2009; Li et al. 2012a). SYTs were originally identified as exocytosis-related proteins in animal cells (Südhof 2002). SYTVII in animals is related to membrane resealing, which takes place via calcium-dependent exocytosis following mechanical disruption of the plasma membrane (Reddy et al. 2001; McNeil and Kirchhausen 2005). Both immunological and genetic approaches have demonstrated that plant SYT1 is also involved in the resealing of plasma membranes damaged by freezing-induced mechanical stress (Yamazaki et al. 2008). During injury, for example, the following series of events takes place: (1) ice crystals spread into the extracellular space and (2) physically press against the plasma membrane; (3) the plasma membrane is eventually mechanically punctured; (4) calcium influx occurs from the extracellular space into the cytoplasm through the damaged sites; and (5) endomembranes are fused at the site of the damaged plasma membrane via calcium-binding SYT1. These events eventually seal the damaged site and decrease the occurrence of freezing injury.

Cold acclimation induces an increase in endocytosis-related proteins of the DRP family and clathrin heavy chains in DRM fractions (Minami et al. 2009, 2010). In these proteins, DRP1E is also transcriptionally regulated by low temperature, whereas other DRP genes are not greatly induced by cold treatment (Minami et al. 2015). A genetic approach using *drp1e* mutants has demonstrated that DRP1E does not affect freezing tolerance before cold acclimation but is needed for full development of freezing tolerance afterwards. According to microscopic analysis, DRP1E localizes nonuniformly in specific areas of the plasma membrane; furthermore, dot-like GFP–DRP1E signals are observed that do not move horizontally but instead appear and disappear from the cell surface. These results support the hypothesis that DRP1E functions to accelerate endocytotic events on the plasma membrane, rearranges plasma membrane components during cold acclimation via the clathrin-dependent endocytosis pathway, and eventually enhances freezing tolerance (Minami et al. 2015).

PLDδ, a lipid modification enzyme, increases in DRM fractions during cold acclimation (Kawamura and Uemura 2003; Minami et al. 2009). Several recent studies mainly using genetic approaches have revealed that PLDs are also strongly associated with plasma membrane stability and freezing tolerance during cold acclimation. The regulation of phospholipid metabolism during cold acclimation is one of the most well-studied mechanisms of plasma membrane lipids. PLD produces phosphatidic acid via hydrolysis of membrane phospholipids. Experimental evidence exists that PLDδ acts as a positive regulator of plant freezing tolerance. Although cold-induced PLDδ seems to be involved in neither the expression of various cold-regulated proteins nor an increase in sugars, which are known to be important components in freezing tolerance acquisition in plants, experimental evidence exists that PLDδ acts as a positive regulator of plant freezing tolerance (Li

et al. 2004). Cytosolic acyl-coenzyme A-binding protein 6 (ACBP6) enhances freezing tolerance in conjunction with the accumulation of some phosphatidic acid species and an increase in *PLDδ* expression, which suggests that ACBP6 is involved in the induction of the *PLDδ* gene (Chen et al. 2008). The *pldα1* mutant, however, increases freezing tolerance, most likely as the result of higher accumulation of COR47, COR78, and osmolytes during cold acclimation (Rajashekar et al. 2006). These observations may reflect the potential roles of PLDs in cold acclimation processes, including phosphatidic acid signaling.

4.3.4 GPI-Anchored Proteins Responded to Cold Acclimation

GPI-anchored proteins are a group of lipid-modified plasma membrane proteins that are anchored to the membrane via glycolipids. Changes in plasma membrane lipids during cold acclimation should thus directly influence the behavior and function of GPI-anchored proteins. GPI-anchored proteins are synthesized in the ER lumen and are always transferred to the extracellular leaflet of the plasma membrane via the vesicular transport system (Udenfriend and Kodukula 1995; Eisenhaber et al. 1999; Ferguson 1999). Diverse responses of GPI-anchored proteins in the juxtamembrane have recently been elucidated (Takahashi et al. 2016b). The abundances of 44 of 163 GPI-anchored proteins are significantly increased in the plasma membrane but are quite stable in DRMs, which suggests that a GPI-anchored protein–microdomain interaction exists in the plasma membrane and that the contact dynamics of these two factors can be changed by cold acclimation treatment.

In regard to the functions of cold acclimation-responsive GPI-anchored proteins, these proteins are more or less associated with cell wall organization and remodeling (Takahashi et al. 2016b). The importance of cell wall characteristics during cold acclimation and freezing stress, especially compositions and pore sizes, has been discussed earlier, as ice nucleation starts in intercellular space (Pearce 1988; Pearce and Fuller 2001; Yamada et al. 2002; Rajashekar et al. 2006). Increases in pectin content and the degree of methyl esterification have been observed in cell walls of oilseed rape and pea (Kubacka-Zębalska and Kacperska 1999; Solecka et al. 2008; Baldwin et al. 2014). Associations of hemicellulose and cuticular wax with cold acclimation and freezing tolerance have also been reported (Zabotin et al. 1998; Amid et al. 2012; Domon et al. 2013). Blue-copper-binding protein (BCB), another cold acclimation-inducible GPI-anchored protein (Takahashi et al. 2016b), has been demonstrated to regulate freezing tolerance via modulation of lignin biosynthesis (Ji et al. 2015). New candidate GPI-anchored proteins identified as cold acclimation-induced plasma membrane proteins (e.g., lipid transfer proteins, LTPs; fasciclin-like arabinogalactan proteins, FLAs; glycerophosphoryl diester phosphodiesterase-like proteins, GPDLs; and O-glycosyl hydrolase family 17 proteins, GH17s) may therefore connect compositional changes of plasma membrane proteomes/lipidomes and cell wall remodeling during cold acclimation via microdomain-dependent or microdomain-independent regulation.

These GPI-anchored cell wall-related proteins are associated with cuticle layer formation (LTPs), cell wall organization and biomechanics (FLAs), cellulose deposition and pectin network formation (GPDLs), and callose turnover (GH17) (Johnson et al. 2003; Levy et al. 2007; Hayashi et al. 2008; DeBono et al. 2009; MacMillan et al. 2010; Kim et al. 2012). As mentioned above, the microdomain component sitosterol-β-glucoside is considered to be a primer of cellulose synthesis on the plasma membrane or a regulating factor of cellulose synthase and its activity, as these activities are enriched in DRM fractions (Peng 2002; Schrick et al. 2004; Endler and Persson 2011). These GPI-anchored cell wall-related proteins may therefore regulate cell wall dynamics via remodeling of the plasma membrane environment and/or microdomains during cold acclimation, an idea based on studies of cellulose synthase. Yeast PER1, required for

lipid remodeling of GPI, is, in fact, important for appropriate targeting of GPI-anchored proteins to microdomains, which suggests that the membrane environment in which GPI is anchored may play significant roles in the function of GPI-anchored proteins (Fujita et al. 2006).

We have identified several GH17s potentially GPI-anchored to the plasma membrane that are induced during cold acclimation in several plant species (Li et al. 2012a; Takahashi et al. 2013a, 2016b). One of these GH17 proteins, AtBG_ppap, is essential for callose turnover and is a key component for regulation of plasmodesmal movement and cell-to-cell communication (Levy et al. 2007). Grison et al. (2015) have recently reported that the plasmodesmata membrane is enriched in sterols and sphingolipids, reminiscent of lipid profiles of DRM fractions. Although not all plasmodesmata-localizing proteins are fractioned to DRM proteins, at least one, namely, plasmodesmata callose binding 1 (PDCB1), is partitioned to DRMs, and its localization is influenced by sterol depletion. Specific partitioning of GPI-anchored proteins to DRMs has not been confirmed (Takahashi et al. 2016b), but these proteins may be connecting with microdomain-enriched plasmodesmata at appropriate times. Interestingly, GH17s in poplar degrade plasmodesmal neck callose to release cell-to-cell communications toward bud dormancy release after winter freezing (Rinne et al. 2001, 2011). Callose-dependent regulation of intercellular communication via plasmodesmata might be coordinated with lipid remodeling of the plasma membrane and/or microdomains during cold acclimation and freezing stress.

4.4 Future Perspectives

Technical advances in omics research should help facilitate the discovery of new aspects of compositional changes of the plasma membrane during cold acclimation. Careful observations and refined analyses of physiological and genetic studies of plasma membrane-associated proteins using their mutants have unveiled the impact of lipidomic and proteomic changes and the importance of the plasma membrane during cold acclimation and freezing (Table 4.1).

Table 4.1 Representative plasma membrane-associated proteins characterized by previous studies

Name	Change[a]	Function	Phenotype[b]		References
			Loss of function	Gain of function	
LCB kinase 1 (LCBK1)	→	Kinase activity of sphingolipid LCB	↓ freezing tolerance		Huang et al. (2017)
			↓ sugar and proline content	↑ freezing tolerance	
			↑ ROS level	↑ sugar and proline content	
			↓ ROS-associated genes		
LCB kinase 2 (LCBK2)	→	Kinase activity of sphingolipid LCB	↓ cold-induced PHP-P synthesis	N/A	Dutilleul et al. (2012)
			↑ root growth at 12 °C		
			↓ DELLA and RGL3 genes		
Sphingolipid Δ8 LCB desaturase (SLD1)	↑ CA3d	Desaturase activity of sphingolipid LCB	↓ Δ8 unsaturation of LCB	N/A	Chen et al. (2012)
			↓ tolerance against prolonged chilling stress		Nagano et al. (2014)
			↓ total sphingolipid and root growth under cold temperature (sld1sld2)		

(continued)

Table 4.1 (continued)

Name	Change[a]	Function	Phenotype[b]		References
			Loss of function	Gain of function	
Sterol glycosyltransferase (SGT)	N/A	Glycosylation of sterol molecules	↓ freezing tolerance	↑ cold tolerance	Mishra et al. (2013), (2015)
			↓ FS and SG	↑ *RD29a* and *RD29b* genes (*A. thaliana* overexpressing *W. somnifera SGT* family gene)	
Lipocalin	↑ CA1d	Transport of small and hydrophobic molecule	N/A	↑ freezing tolerance (protoplast)	Charron et al. (2002)
					Uemura et al. (2006)
Protein phosphatase 2C (PP2C)	↑ CA1d	Serine/threonine phosphatase activity	↑ freezing tolerance	N/A	Tähtiharju and Palva (2001)
		Negative regulator of ABA			
Synaptotagmin 1 (SYT1)	↑ CA1d	Calcium-induced membrane-membrane fusion	↓ freezing tolerance	N/A	Yamazaki et al. (2008)
Dynamin-related protein 1E (DRP1E)	↑ CA12h	Scission vesicle from the plasma membrane	↓ freezing tolerance	N/A	Minami et al. (2015)
Cold-responsive protein kinase 1 (CRPK1)	→	Phosphorylation of 14-3-3 protein and negatively regulates CBFC-repeat binding factor (CBF) expression	↑ freezing tolerance	N/A	Liu et al. (2017)
Phospholipase Dδ (PLDδ)	↑ CA3d	Hydrolyze phospholipids to phosphatidic acid and head group	↓ freezing tolerance	↑ freezing tolerance	Li et al. (2004)
Phospholipase Dα1 (PLDα1)	N/A	Hydrolyze phospholipids to phosphatidic acid and head group	↑ freezing tolerance	N/A	Welti et al. (2002)
			↑ COR47 and COR78		Rajashekar et al. (2006)
			↑ raffinose content		
Blue-copper-binding protein (BCB)	↑ CA7d	Regulator of lignin biosynthesis	↑ freezing tolerance	N/A	Ji et al. (2015)
			↓ phenylalanine ammonia-lyase genes (PALs)		
			↓ lignin content		
Lipid transfer proteins (LTP)	↑ CA3h	Lipid transport	No changes	↑ freezing tolerance	Guo et al. (2013)
				↑ soluble sugar content	

[a]Arrows indicate expression trends at the mRNA and/or protein level during cold acclimation (CA)
[b]Phenotypes of knockdown/knockout or overexpression mutants

On the other hand, most of the previous studies employed single condition for cold acclimation treatment. However, cold acclimation conditions (e.g., processing temperature, cooling rate, and light conditions) can influence plasma membrane changes and eventual plant freezing tolerance. Furthermore, plant freezing tolerance can be determined by cellular responses to not only cold acclimation but also freezing and post freeze-thawing processes. Omics studies of the plasma membrane should therefore be focused not only on cold acclimation-induced changes but also on changes during acclimation, freezing, thawing, and recovery.

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5

Natural Variation in Freezing Tolerance and Cold Acclimation Response in *Arabidopsis thaliana* and Related Species

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Abstract

During low-temperature exposure, temperate plant species increase their freezing tolerance in a process termed cold acclimation. The molecular mechanisms involved in cold acclimation have been mostly investigated in *Arabidopsis thaliana*. In addition, other Brassicaceae species related to *A. thaliana* have been employed in recent years to study plant stress responses on a phylogenetically broader basis and in some cases with extremophile species with a much higher stress tolerance. In this paper, we briefly summarize cold acclimation responses in *A. thaliana* and current knowledge about cold acclimation in *A. thaliana* relatives with special emphasis on *Eutrema salsugineum* and two closely related *Thellungiella* species. We then present a transcriptomic and metabolomic analysis of cold acclimation in five *A. thaliana* and two *E. salsugineum* accessions that differ widely in their freezing tolerance. Differences in the cold responses of the two species are discussed.

Keywords

Arabidopsis thaliana · Cold acclimation · *Eutrema salsugineum* · Gene expression · Metabolomics · Transcriptomics

Abbreviations

AGI	*Arabidopsis* gene identifier
AP2	Apetala2
CBF	C-repeat binding factor
COR	Cold regulated
DH	Doubled haploid
DREB	Dehydration-responsive element binding
ERF	Ethylene response factor
GC-MS	Gas chromatography-mass spectrometry
GWAS	Genome-wide association study
ICA	Independent components analysis
ICE	Inducer of CBF expression
LT_{50}	Lethal temperature of 50% electrolyte leakage
QTL	Quantitative trait loci
RIL	Recombinant inbred line
RNA-Seq	RNA sequencing

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5.1 Freezing Tolerance and Cold Acclimation in *Arabidopsis thaliana*

Extreme temperatures can damage sensitive plant tissues and impair physiological function. When temperatures drop below 0 °C, ice may crystallize in the extracellular spaces of exposed tissues such as leaves. This leads to cellular dehydration as water is drawn from the cells to the growing ice crystals (Pearce and Willison 1985). The extent of this freeze-induced dehydration is a direct function of the sub-zero temperature. Consequently, in plants that are not chilling sensitive, freezing damage is mainly the result of increasing water loss from the cells with decreasing temperature. The primary sites of this damage are the cellular membranes (Steponkus 1984), although freeze-induced inactivation of enzymes has also been observed (Thalhammer et al. 2014a).

In addition to their constitutive (non-acclimated) freezing tolerance, plants native to temperate and boreal climates show an increase in their freezing tolerance during fall (Preston and Sanve 2013). This process that can take from a few days to several weeks, depending on temperature regime and plant species, is termed cold acclimation (Levitt 1980). Cold acclimation has been extensively studied, and it has been shown that it is a multigenic, quantitative trait involving massive reprogramming of the transcriptome, proteome and metabolome (see Guy et al. 2008; Thomashow 2010; Hincha et al. 2012 for reviews). *Arabidopsis thaliana* has for many years been the main model system for the investigation of the molecular basis of freezing tolerance due to its small genome, a rapid life cycle, the sequenced genome and availability of comprehensive genetic tools.

Forward and reverse genetics approaches together with transcript profiling data suggest the induction of multiple transcriptional pathways during cold acclimation (Chinnusamy et al. 2007). In addition, the identification of quantitative trait loci (QTL) for freezing tolerance after cold acclimation in *A. thaliana* recombinant inbred line (RIL) populations generated from crosses between accessions with high variation in freezing tolerance such as Can-0 x Col-0, Bur-0 x Col-0, Te-0 x C24 or L*er* x Cvi (Alonso-Blanco et al. 2005; Gery et al. 2011; Meissner et al. 2013) resulted in different candidate genes or genomic regions depending on the accessions involved. Currently, the best understood gene regulatory network involved in cold acclimation depends on the C-repeat binding factor (CBF) family of apetala2 (AP2)-type transcriptional activators. *CBF* genes appear to be ubiquitous in plants and are almost always present in multiple copies (Chinnusamy et al. 2007; Thomashow 2010; Shi et al. 2015). *CBF* genes were also found to be responsible for freezing tolerance QTL in the L*er* x Cvi population (Alonso-Blanco et al. 2005) and in a RIL population derived from accessions from Italy and Sweden (Oakley et al. 2014). In both cases a deletion in either the promoter or coding region of *CBF2* is responsible for the lower freezing tolerance of the Cvi and Italy accessions, respectively (Alonso-Blanco et al. 2005; Gehan et al. 2015). In other QTL mapping studies (Gery et al. 2011; Meissner et al. 2013) and in a recent genome-wide association study (GWAS, Horton et al. 2016), however, other genomic regions were identified as being important for higher freezing tolerance. In addition, a study comparing the freezing tolerance of 54 *A. thaliana* accessions under both non-acclimating and acclimating conditions found no correlation between the expression levels of *CBF1*, *CBF12* or *CBF13* and freezing tolerance (Zuther et al. 2012). On the other hand, ectopic expression of one of the *CBF* genes in *A. thaliana* leads to an increase in freezing tolerance in the absence of cold treatment (Artus et al. 1996; Jaglo-Ottosen et al. 1998; Kasuga et al. 1999; Gilmour et al. 2004), and a knock-out of all three *CBF* genes renders plants incapable of a cold acclimation response that increases plant freezing tolerance (Zhao et al. 2016), attesting to the central importance of this signal transduction pathway.

Downstream of the CBF transcription factors, more than 100 genes constitute the CBF regulon of cold-regulated genes (Hannah et al. 2005; Thomashow 2010). While this regulatory

pathway is well studied, only limited knowledge is available about the functional role of the proteins encoded by these cold-regulated genes. However, for some it is known that they encode enzymes responsible for the biosynthesis of compatible solutes (Gilmour et al. 2000; Cook et al. 2004; Maruyama et al. 2009). One of these solutes is the trisaccharide raffinose, whose content is also highly correlated with freezing tolerance in cold-acclimated Arabidopsis accessions (Zuther et al. 2012). However, when raffinose accumulation during cold acclimation is blocked by an inactivation of the *Raffinose Synthase* gene, this does not result in a reduction in freezing tolerance after cold acclimation. This indicates that compatible solutes form a redundant system of cellular protection that does not rely on any specific compound.

The only other CBF target genes whose function has been investigated in detail are *COR15A* (*cold-regulated 15A*) and *COR15B* which form a tandem repeat in the *A. thaliana* genome (Wilhelm and Thomashow 1993). Both proteins are nuclear encoded and are post-translationally imported into the chloroplast stroma (Lin and Thomashow 1992; Nakayama et al. 2007; Candat et al. 2013). They increase the non-acclimated freezing tolerance of leaves upon overexpression (Artus et al. 1996; Thalhammer et al. 2014a), and a simultaneous knock-down of both genes strongly reduces acclimated freezing tolerance in *A. thaliana* (Thalhammer et al. 2014a), indicating that the proteins are necessary to attain full freezing tolerance. COR15A and COR15B are intrinsically disordered in dilute solution but fold into α-helices upon gradual water loss or in the presence of high concentrations of co-solutes as would be encountered in the cells of partially frozen leaves (Thalhammer et al. 2010, 2014a; Bremer et al. 2017a, b). Both proteins are able to prevent freeze-thaw damage to liposomes (Thalhammer et al. 2014a; Bremer et al. 2017b) in agreement with reduced electrolyte leakage from leaves of overexpression lines and increased leakage from knock-down lines indicating altered membrane stability (Steponkus et al. 1998; Thalhammer et al. 2014a).

Another group of compounds that play an important role in plant freezing tolerance are flavonoids. The flavonoid biosynthetic pathway is cold induced at the transcriptional level (Hannah et al. 2006; Korn et al. 2008; Schulz et al. 2015), independent of the CBF signal transduction pathway. The content of several flavonols and anthocyanins is correlated with leaf freezing tolerance after cold acclimation (Korn et al. 2008; Schulz et al. 2015). In addition, several mutants in the biosynthetic pathway are impaired in their freezing tolerance, while a line constitutively overexpression a transcription factor (PAP1/MYB75) activating the pathway shows increased freezing tolerance (Schulz et al. 2016). These data show that both CBF-dependent and CBF-independent pathways functionally contribute to *A. thaliana* freezing tolerance and cold acclimation. QTL mapping and GWAS results suggest that more contributors remain to be discovered and functionally characterized.

5.2 Freezing Tolerance and Cold Acclimation in *Arabidopsis thaliana* Relatives

5.2.1 Relatives of *A. thaliana* That Have Been Used in Cold Acclimation Research

Over the past years, the focus of many researchers has widened from *A. thaliana* to include in addition several closely related relatives of this species. A prerequisite for this development was that the phylogenetic position of *A. thaliana* and its close relatives has been resolved (Al-Shehbaz et al. 1999; Koch et al. 2008; Koch and German 2013), including the renaming of several species. While this development has resolved the phylogenetic relationships among these species, it also makes it sometimes difficult to work with the older literature, as species names have often been erroneously used (and confused) over the years.

The *A. thaliana* relatives that have mainly been used in plant stress tolerance research include about ten species within the genus

Arabidopsis native to Eurasia, North Africa and North America, such as *A. lyrata*, *A. halleri* and *A. arenosa*, which are species groups representing three major evolutionary lineages and are diploids with 2n = 16 in contrast to *A. thaliana* with 2n = 10 (Mitchell-Olds 2001; Koch et al. 2008). In addition, these species are mainly outcrossing, in contrast to *A. thaliana*, resulting in higher levels of genetic variation within populations (Mitchell-Olds 2001). Another relative, *Arabis alpina* as type species of the genus *Arabis*, also has 2n = 16 chromosomes and is more distantly related to *A. thaliana.* It is found in Eurasia, East Africa, Northern California and Southern Oregon (Mitchell-Olds 2001). A further natural *Arabis* group with 2n = 14 chromosomes from North America has been reclassified as the genus *Boechera* and is more closely related to *A. thaliana* than to the group of species with 2n = 16 chromosomes (Mitchell-Olds 2001). In addition, also species such as *Cardamine impatiens* and *C. resedifolia*, *Capsella bursa-pastoris* and *Thlaspi arvense*, a winter annual weed surviving temperatures down to −40 °C (Warwick et al. 2002), have been used to investigate molecular differences between *A. thaliana* and its wild relatives with regard to cold acclimation and freezing tolerance (Zhou et al. 2007; Ometto et al. 2012, 2015; Kasianov et al. 2017). Research was furthermore extended to agronomically important crucifers such as *Brassica* spp. and radish (*Raphanus* spp.), which are widely distributed in Eurasia (Teutonico et al. 1995; Jaglo et al. 2001; Gao et al. 2002; Kole et al. 2002; Liu et al. 2017).

Some extremophile relatives of *A. thaliana* can be found in the genus *Thellungiella* from the Brassicaceae family. The species in this genus that have attracted most attention of researchers include *Eutrema salsugineum*, which was previously called *Thellungiella salsuginea* (Al-Shehbaz et al. 1999; Amtmann 2009), as well as *T. halophila*, *T. botschantzevii* and *T. parvula*, the latter recently renamed to *Schrenkiella parvula* (Koch and German 2013). For *E. salsugineum* several accessions spanning a large geographical range from the USA and Canada through China and Russia are available (Lee et al. 2012b), and their phylogenetic relationships have been investigated in detail (Wang et al. 2015).

5.2.2 Freezing Tolerance and Cold Acclimation in *A. thaliana* Relatives Other Than *Eutrema* spp.

Constant environmental pressure including abiotic and biotic stress factors result in natural selection for stress tolerance causing ecologically important polymorphisms in the genomes of extremophile *A. thaliana* relatives (Mitchell-Olds 2001). However, research on non-model taxa has focused until recently on phylogenetics and genomic comparisons, while information on stress physiology and functional adaptations was rare (Clauss and Koch 2006). Differences between the annual model species *A. thaliana* with limited stress tolerance traits and its more stress tolerant perennial relatives provide possibilities for the discovery of novel molecular regulations and/or metabolic mechanisms underlying stress tolerance and the repeated switch from vegetative to reproductive growth. In particular the relationship between such life history traits and stress tolerance has so far been hardly explored.

Natural variation within species and the consideration of latitudinal and climatic gradients have been used to understand cold acclimation and differences in freezing tolerance in *A. thaliana* (Hannah et al. 2006; Zhen and Ungerer 2008a, b; Zuther et al. 2012; Schulz et al. 2015; Horton et al. 2016) and its wild relatives. Two *Cardamine* species from either high (*C. resedifolia*) or low altitudes (*C. impatiens*) in the South-Eastern Alps were investigated for their distinct stress responses (Ometto et al. 2012). High-throughput sequencing in combination with comparisons to the *A. thaliana* genome sequence was used to identify putative cold-responsive genes. A high habitat-specific selection pressure was identified as responsible for a faster evolution of cold-responsive genes in the high-altitude species *C. resedifolia* compared

to *C. impatiens* (Ometto et al. 2012). A further investigation across 10 populations of each of the 2 species analysing genetic variability of 19 cold-responsive genes indicated less gene flow in high-altitude versus low-altitude populations and no relevant roles in the evolutionary adaptation to alpine environments within species (Ometto et al. 2015). Frost tolerance of Northern populations varied strongly when seeds of plant families from eight populations of North American *A. lyrata* were collected along a latitudinal gradient. Higher frost tolerance of higher latitude populations was correlated with smaller plant size which was hypothesized to limit species distribution at the Northern limits of species habitats (Wos and Willi 2015).

Arabidopsis relatives share elements of cold-response pathways with *A. thaliana*, as was shown, for example, for three cold-induced bZIP transcription factors in *B. oleracea* (Hwang et al. 2016) or *dihydroflavonol 4-reductase* (*DFR*) genes in *B. rapa* encoding an enzyme of the cold-induced anthocyanin pathway (Ahmed et al. 2014). In addition, the rapid accumulation of *CBF*-like genes was followed by expression of the *Bn115* gene, an ortholog of the *A. thaliana* CBF-regulated *AtCOR15A* in *B. napus* (Jaglo et al. 2001). Three *BnCBF* genes of *B. napus* cv. Jet Neuf are similar to *AtCBF1*, transcripts of a fourth *AtCBF*-like gene, *BnCBF17*, increased in abundance shortly after cold exposure and induced the expression of *Bn28*, an ortholog of *AtCOR6.6* (Gao et al. 2002). Investigation of gene families in *A. thaliana* relatives also leads to the identification of new regulators of the cold response as shown for the brassinazole-resistant (BZR) transcription factors, known as positive regulators of brassinosteroid signal transduction that were suggested to transcriptionally activate the CBF cold-responsive pathway via the inducer of CBF expression (ICE) transcription factor (Saha et al. 2015). These similarities facilitate cross-species microarray analysis as one method to differentiate between cold stress responses of relatives with different freezing tolerance, e.g. *T. arvense* with a freezing tolerance almost twice as high as that of *A. thaliana* Col-0 after 3 weeks of cold acclimation (Sharma et al. 2007). A similar response as in *A. thaliana* at the very early stage of cold acclimation was detected with some quantitative and timing differences for some cold-regulated genes. One reported difference was the increased production of S-adenosyl-methionine, a methyl donor originating from sulphur assimilation (Sharma et al. 2007). One disadvantage of cross-species analysis by microarrays is the limitation to already known genes with sufficient sequence homology to allow hybridization (Lee et al. 2013). These problems can be overcome by the development of species-specific microarrays. This strategy was followed by assembling a 135K oligonucleotide array from a genome-wide transcriptome analysis of two *B. rapa* doubled haploid (DH) lines (Chiifu and Kenshin) of contrasting freezing tolerance. Cold treatment induced 885 and 858 genes in the Chiifu and Kenshin lines with various transcription factors differentially expressed between the lines. Timing of gene expression seemed to be more important for freezing tolerance than gene expression levels since expression of known cold-responsive genes did not correlate with freezing tolerance (Jung et al. 2014). Another possibility is the use of sequencing-based transcriptomics (RNA-Seq). Illumina sequencing was also used to investigate stress-responsive cis-regulatory divergence in *A. thaliana* x *A. lyrata* and *A. thaliana* x *A. halleri* hybrids. *A. lyrata* colonizes also northern parts of Europe, while the habitat of *A. halleri* is restricted to Central Europe. Only *A. lyrata* but not *A. halleri* showed an allele-specific enrichment of cis-acting expression differences among cold-responsive genes in *A. thaliana* (He et al. 2016). High constitutive expression of cold-responsive genes and high constitutive freezing tolerance were also found in a single genetic lineage of *A. arenosa* colonizing railways throughout Europe in comparison to other lineages from mountain regions (Baduel et al. 2016). RNA-Seq was also used to characterize *AP2/ERF* (*ethylene response factor*) genes and their role in the cold response of *B. napus*. Members of the *RAV* and *dehydration-responsive element-binding* (*DREB*) subfamilies were expressed early after cold treatment compared to the *AP2* subfamily (Du et al. 2016).

Transcriptome profiling of cold stressed siliques during pod-filling stages in Indian mustard (*B. juncea*) identified 283 core cold-induced transcripts as well as limited induction of genes encoding protein kinases only during early silique development (Sinha et al. 2015).

A. thaliana relatives are also an interesting tool to study the role of polyploidization and the subsequent neofunctionalization of duplicated genes in cold responses and freezing tolerance. One example for such studies is *C. bursa-pastoris*, a recent allotetraploid. A high-quality assembly of the genome and a transcriptome atlas have recently been reported (Kasianov et al. 2017). Promoter comparisons of homologous gene pairs with different expression levels pointed to an asymmetry of regulatory elements with main roles in stress adaptation, e.g. binding sites for CBF transcription factors controlling cold acclimation responses (Kasianov et al. 2017). A genome-wide analysis of the *CBF/DREB1* gene family from *B. rapa* revealed ten *BrDREB1* homologs with nine of them derived from a genome triplication in the tribe Brassicaceae. Eight selected *BrDREB1* genes are cold-responsive and might provide additional functions in cold stress responses compared to *A. thaliana* (Lee et al. 2012a).

An analysis of 108 inbred lines of a cross between two genotypes of *Boechera stricta*, a highly adapted perennial species from regions with low winter temperatures (Heo et al. 2014), identified one main QTL for freezing tolerance from an outdoor experiment but also for short and long photoperiod conditions in growth chamber experiments. This QTL showed co-localization with photosynthetic performance QTL under all conditions. It was syntenic to the *AtCBF* locus, thus identified variation in *CBF* genes as an important mechanism for variation in freezing tolerance (Heo et al. 2014). Six QTL for winter survival, non-acclimated and acclimated freezing tolerance and flowering time were detected during multiple winters in immortalized populations of oilseed *B. rapa* (RILs) and *B. napus* (DH lines) derived from crosses of annual and biennial types. Some alleles contributed to improved acclimated freezing tolerance, better winter survival and delayed flowering time with allelic variation in both species (Kole et al. 2002). QTL with significant effects on freezing tolerance were missing in a doubled haploid line population of *B. napus*, whereas in an F2 population of *B. rapa*, four QTL were linked to acclimated freezing tolerance and acclimation ability, and two regions were associated with freezing tolerance under non-acclimated conditions (Teutonico et al. 1995).

A. thaliana relatives are also used for the analysis of processes involved in cross protection to different stresses, e.g. an investigation of the epigenetic basis of elevated heat tolerance in cold-acclimated *B. rapa* (Liu et al. 2017). Here, methylation and demethylation during cold acclimation resulted in over 1500 differentially methylated genes and affected two significantly altered pathways, malate dehydrogenase activity and carbon fixation.

In addition, insights into different molecular strategies for cold adaptation have also come from metabolic analyses of different populations of Cruciferae. Geographically separated populations of *A. lyrata* sp. *petraea* from Wales to Iceland revealed distinct metabolic phenotypes that were mainly determined by sugar and amino acid composition. However, there were no latitudinal gradients for the concentrations of these metabolites in the plants (Davey et al. 2009). On the other hand, in a study with eight accessions of the alpine perennial *A. alpina* from altitudes between approximately 2000 and 3000 m above sea level, enhanced sugar accumulation with increasing altitude was observed. This was accompanied by lower chlorophyll loss in response to chilling and an inhibition of stress-induced leaf senescence under cold conditions (Wingler et al. 2015).

Finally, the *A. thaliana* relatives are good tools for the identification and isolation of genes encoding protective proteins that either vary in sequence and function from homologous genes in *A. thaliana* or are not present there at all. In addition, signal transduction pathways or gene regulatory networks may be more simple and therefore easier to transfer into crop plants than the *A. thaliana* counterparts. One example for the

latter case is the single copy *CBF* gene in *T. arvense* compared to three tandem copies in *A. thaliana*. An overexpression of *TaCBF* in *A. thaliana* resulted in a constitutive freezing tolerance comparable to plants under cold-acclimated conditions (Zhou et al. 2007). Other examples for overexpression of cold-responsive genes from *A. thaliana* relatives are transgenic tobacco plants ectopically expressing *C. bursa-pastoris COR15B* displaying higher tolerance when exposed to chilling and freezing temperatures (Wu et al. 2012b) similar to the expression of *AtCOR15B* in *A. thaliana* (Thalhammer et al. 2014a). Similarly, increased tolerance to chilling and freezing combined with dwarfism and delayed flowering was observed after overexpression of *CBF* from *C. bursa-pastoris* in tobacco (Zhou et al. 2014). Overexpression of the *RsICE1* transcription factor from *Raphanus sativus* triggered the cold-response networks via CBF/DREB1 transcription factors in rice and enhanced low-temperature tolerance accompanied by higher sugar, proline and chlorophyll levels and strong upregulation of the downstream cold-regulated genes *OsDREBL* and *OsTPP1* (Man et al. 2017). Furthermore, the *Rare Cold-Inducible 35* gene from *C. bursa-pastoris* (*CbRCI35*), which belongs to the type III peroxidase gene family, activated cold-responsive signalling and improved low-temperature tolerance after heterologous expression in tobacco by modulating reactive oxygen species (ROS) homeostasis (Zhou et al. 2016).

5.2.3 Freezing Tolerance and Cold Acclimation in *Eutrema* spp.

The most important *A. thaliana* relative that has been used in research on cold acclimation and freezing tolerance is *E. salsugineum*, an extremophile with natural high tolerance to multiple stresses (Bressan et al. 2001) and a closer relation to agronomically important *Brassica* species (Wong et al. 2005; Bailey et al. 2006). Previous research on abiotic stress responses in *E. salsugineum* discovered mechanisms important for potential adaptation to extreme stress conditions, constitutive preparation for stress conditions and a lower sensitivity combined with the requirement of higher stress doses for induction of transcriptional responses (Amtmann 2009). Interestingly only a small overlap of expressed genes, most of them with unknown function, was found when investigating *E. salsugineum* Yukon accession under cold, drought and salinity conditions indicating specific responses to individual stresses (Wong et al. 2005).

Since the nuclear genomes of *E. salsugineum* (Wu et al. 2012a; Yang et al. 2013) and of *T. parvula* (Dassanayake et al. 2011) as well as the mitochondrial genome of *T. parvula* (Wang et al. 2016) were fully sequenced, both species have become interesting resources for the discovery of novel stress response mechanisms which are lacking in the non-extremophile species *A. thaliana*. The sequencing of the *E. salsugineum* genome has been complemented by a de novo transcriptome assembly based on 454 pyrosequencing and the development and validation of a genome-wide, commercially available 44 k Agilent oligonucleotide microarray based on the Yukon accession (Lee et al. 2013). Approximately 25% of the transcript sequences in this transcriptome assembly are unique to *E. salsugineum*, and a detailed annotation of late embryogenesis abundant (LEA) proteins, mitogen activated protein (MAP) kinases and protein phosphatases was provided (Lee et al. 2013). Recent studies on transcriptional changes in leaves and roots of the *E. salsugineum* Shandong accession using RNA-Seq identified several genes encoding transcription factors together with five aquaporin genes and typical cold-responsive genes such as *EsCOR47* and *EsICE1* as differentially expressed after 24 h exposure to low temperature (Wang et al. 2017). In addition, an enrichment of cold-responsive genes was found in metabolism, photosynthesis, circadian rhythm and transcriptional regulation. An involvement of jasmonic acid and repressed photosynthetic processes, but high tolerance to photoinhibition, were described as part of the cold response of this species (Wong et al. 2006).

Investigation of the freezing tolerance of *Eutrema* spp. revealed no extremophile character of this and closely related species, although three accessions of *E. salsugineum* from Russia (Tuva, Altai 1 and Altai 2) and one accession each from the *Thellungiella* species *T. halophila* (Bayanaul from Kazakhstan) and *T. botschantzevii* (Saratov from Russia) showed higher freezing tolerance than the most tolerant *A. thaliana* accession N14 from Russia (Lee et al. 2012b). Additionally *E. salsugineum* shows a remarkable long-term acclimation capacity, with a significant increase in freezing tolerance after 3 weeks of cold acclimation compared to 2 weeks. Such an increase was not observed in *A. thaliana* (Khanal et al. 2015).

Evidence for different metabolic low-temperature acclimation strategies was obtained when 14 accessions of *E. salsugineum* and one each of *T. halophila* and *T. botschantzevii* were compared with 54 *A. thaliana* accessions (Lee et al. 2012b; Zuther et al. 2012). While raffinose content is highly correlated with acclimated freezing tolerance in *A. thaliana*, *Eutrema* spp. accumulate very low amounts of this sugar. However, *Eutrema* spp. plants accumulate higher amounts of proline, which are correlated with acclimated freezing tolerance in these species but not in *A. thaliana* (Lee et al. 2012b). The same difference between these species was also found under salt stress (Kazachkova et al. 2013; Lee et al. 2016) indicating a general metabolic difference in the stress responses of the different species. A comprehensive study comparing transcript and metabolite profiles of *E. salsugineum* Yukon plants either grown under controlled conditions or in the field detected metabolic plasticity depending on growth conditions. Plants grown in their native Yukon habitat, which is characterized by nitrogen-poor soil, showed low levels of proline under salt stress, while plants grown under controlled conditions showed high or low proline levels under salt stress depending on nitrogen availability (Guevara et al. 2012).

Proteomic studies of leaves of *E. salsugineum* using two-dimensional electrophoresis found the highest induction of proteins under cold stress after long-term cold acclimation (up to 24 days). Proteins involved in photosynthesis, RNA metabolism, defence response, energy, protein synthesis, folding and degradation, cell wall and cytoskeleton and signal transduction were overrepresented among the cold-regulated proteins (Gao et al. 2009).

5.3 Transcriptomic and Metabolomic Characterization of Cold Acclimation in Different Accessions of *A. thaliana* and *E. salsugineum*

As described above, there is a continuum in the freezing tolerance of *A. thaliana* and *E. salsugineum* when a range of natural accessions is considered. However, the most freezing tolerant *E. salsugineum* accessions are superior in their freezing tolerance to the most tolerant *A. thaliana* accessions (Lee et al. 2012b). We have therefore selected five *A. thaliana* and two *E. salsugineum* accessions (Fig. 5.1) for a transcriptomic and metabolomic analysis to gain deeper insight into the molecular basis of freezing tolerance in these two species. Freezing tolerance was determined by electrolyte leakage assays (Thalhammer et al. 2014b) with detached leaves after a cold acclimation period of 14 days at constant 4 °C. The LT_{50} (lethal temperature of 50% electrolyte leakage) values shown in Fig. 5.1 were taken from (Zuther et al. 2012) for *A. thaliana* and from (Lee et al. 2012b) for *E. salsugineum*.

For the transcriptomic study, we have used Agilent microarrays (http://www.genomics.agilent.com/) specific for *A. thaliana* (V4) and *E. salsugineum* (Lee et al. 2013). We have used these arrays previously to study the transcriptomic responses of the two species to salt stress (Lee et al. 2016). All technical details of sample preparation, array hybridization and data analysis have been described extensively in these two publications. The raw data can be found at the

Accession	LT_{50} (°C)	Differentially expressed probes (unique AGI) FDR p<0.05, log_2 FC >|1|		
		Up-regulated	Down-regulated	Total
Tuva	-15.2	4494 (2187)	4502 (1953)	8996 (4140)
Yukon	-11.7	4574 (2078)	5674 (2393)	10248 (4471)
N13	-12.1	2895 (2225)	3170 (2358)	6065 (4583)
Ms-0	-11.9	1501 (1203)	1388 (1042)	2889 (2245)
Col-0	-9.7	1121 (938)	1281 (982)	2402 (1920)
Can-0	-5.8	514 (438)	709 (552)	1223 (990)
Sah-0	-5.4	1022 (832)	1073 (843)	2095 (1675)

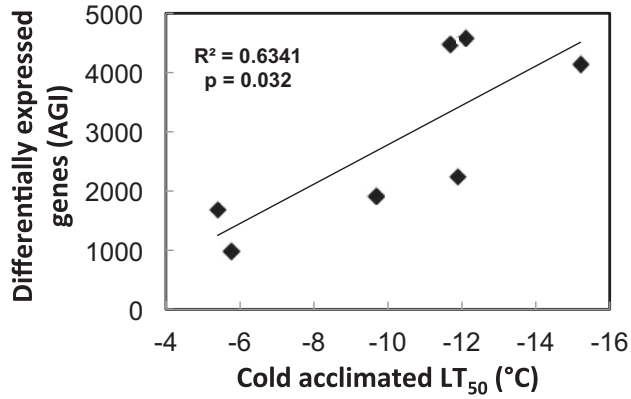

Fig. 5.1 Analysis of differential gene expression during cold acclimation in *A. thaliana* and *E. salsugineum* accessions. The upper panel summarizes the freezing tolerance (as LT_{50} from electrolyte leakage measurements with detached leaves) after 14 days of cold acclimation at 4 °C as reported previously for *A. thaliana* (Zuther et al. 2012) and *E. salsugineum* (Lee et al. 2012b) and the number of significantly (FDR $p < 0.05$, log_2 fold change >|1|) cold-regulated probe sets on the microarrays for two *E. salsugineum* (Tuva, Yukon) and five *A. thaliana* (N13, Ms-0, Col-0, Can-0, Sah-0) accessions. The numbers in parentheses denote the numbers of probe sets that correspond to a unique AGI code in the *A. thaliana* genome. The bottom panel shows the correlation between the LT_{50} of the different accessions from both species after cold acclimation and the number of significantly cold-regulated genes

Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under the accession number GSE100108. Briefly, samples of rosette leaves were obtained from three independent biological experiments. Expression values were considered significantly different at a log_2 fold change above |1| with a p-value <0.05 after false discovery rate correction (Benjamini and Hochberg 1995).

Figure 5.1 shows the number of probe sets on the arrays that were significantly cold regulated after 14 days at a constant temperature of 4 °C and in parentheses the number of significantly regulated probe sets that correspond to a unique annotated gene (unique AGI) in the *A. thaliana* genome. While the differences between these numbers are quite small for *A. thaliana*, they are substantial for *E. salsugineum*. This is due to the fact that the *A. thaliana* array is largely designed based on the corresponding genome sequence, while the *E. salsugineum* array is based on transcriptome sequences from this species (Lee et al. 2013). As noted previously, almost 90% of the unigenes derived from the *E. salsugineum* transcriptome could be aligned to the *E. salsugineum* genome sequence, while only about 75% had orthologous genes in the *A. thaliana*

genome (Lee et al. 2013). As noted also for salt-regulated genes (Lee et al. 2016), the fact that only about half of the cold-regulated genes in *E. salsugineum* could be assigned to orthologous genes in *A. thaliana* indicates that a large proportion of stress-regulated genes in *E. salsugineum* are unique to this species.

Interestingly, however, we observed a significant correlation between the freezing tolerance after cold acclimation and the number of significantly cold-regulated genes across all investigated genotypes from both species (Fig. 5.1). A similar observation has been made previously when nine different *A. thaliana* accessions were compared using Affymetrix arrays (Hannah et al. 2006). This indicates that on the transcript level, freezing tolerance is determined by quantitative differences in the cold response of a large number of genes. Interestingly, a recent RNA-Seq study using the *E. salsugineum* accession Shandong (Wang et al. 2017), which is less freezing tolerant than the accessions studied here (Lee et al. 2012b), also revealed smaller numbers (1091 down, 1691 up) than in the more freezing tolerant accessions investigated here. However, the two studies are difficult to compare because in addition to the different method to determine gene expression levels, this study analysed gene expression after a much shorter cold acclimation time (24 h) and under a different low-temperature regime (8 °C during the day (16 h), 4 °C during the night (8 h)).

However, although similar numbers of genes that could be assigned to an AGI were up- or downregulated in the most freezing tolerant *A. thaliana* accession N13 and in the two similarly freezing tolerant *E. salsugineum* accessions (Fig. 5.1), only approximately one third of the up-regulated and half of the downregulated genes that were identified in at least one accession in the two species were identical between *A. thaliana* and *E. salsugineum* (Fig. 5.2). This indicates that even for the majority of cold-regulated genes that are orthologous between the species, cold regulation was different. The same conclusion was reached in our previous study on salt stress responses of these two species (Lee et al. 2016).

To explore this further on a functional level, we performed an over-/underrepresentation analysis, using the functional bins of the MapMan annotation (Thimm et al. 2004; Lohse et al. 2010). For this purpose we divided the genes that were significantly up- or downregulated during cold acclimation into three groups: *E. salsugineum*-specific, *A. thaliana*-specific and those found in both species (overlap; Fig. 5.3). The numbers of genes in the respective groups can be seen in Fig. 5.2. In total, there were ten bins that showed only overrepresentation of up- or downregulated genes from one species (e.g. *E. salsugineum*-specific up-regulated genes in the bins Xenobiotics, RNA, Protein, Signalling and Development and downregulated genes in the

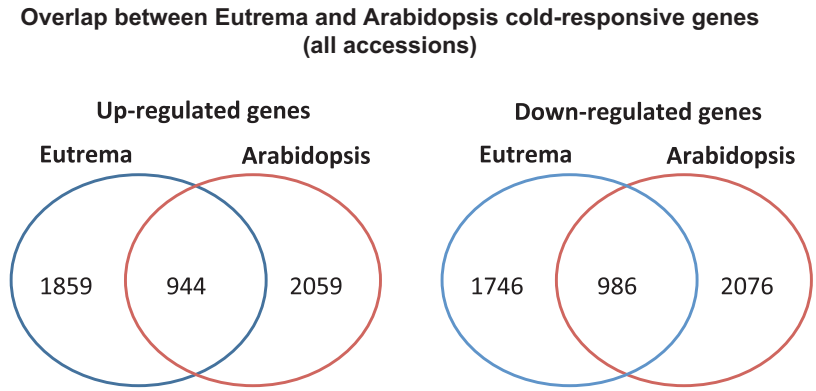

Fig. 5.2 Overlap of cold-regulated genes between *A. thaliana* and *E. salsugineum*. Venn diagrams show the numbers of genes that were either significantly up- or downregulated after 14 days of cold acclimation in at least one *E. salsugineum* or *A. thaliana* accession

Bin #	Bin name	Eutrema-specific up	Arabidopsis-specific up	Overlap up	Eutrema-specific down	Arabidopsis-specific down	Overlap down
1	Photosynthesis						
2	Major CHO						
4	Glycolysis						
5	Fermentation						
8	TCA cycle						
10	Cell wall						
11	Lipid						
13	Amino acids						
14	S-assimilation						
15	Metal handling						
16	Secondary metabolites						
17	Hormones						
19	Tetrapyrrole						
20	Stress						
21	Redox						
23	Nucleotides						
24	Xenobiotics						
27	RNA						
28	DNA						
29	Protein						
30	Signalling						
31	Cell						
33	Development						
34	Transport						

Fig. 5.3 Over-/underrepresentation analysis of genes significantly changed in expression during cold acclimation. Genes were grouped in MapMan bins and overrepresentation or underrepresentation was determined using the Fisher exact test (FDR $p < 0.05$) for genes showing either significant up- or downregulation of expression either specifically in *E. salsugineum* or in *A. thaliana* or in both species (compare Fig. 5.2). Blue boxes indicate significant overrepresentation of up- or downregulated genes in the respective functional bin, while red boxes indicate significant underrepresentation

bins S-assimilation, Signalling and Cell). There were only three bins that were specifically overrepresented among cold-regulated *A. thaliana* genes, namely, Stress among up-regulated and Xenobiotics and Transport among downregulated genes. There were four bins (Glycolysis, Fermentation, TCA cycle, Metal handling) that showed only an overrepresentation of up-regulated genes common to both species. In combination with one bin (Transport) with an overrepresentation of up-regulated genes specific to either and both species and one bin (DNA) with an underrepresentation of both up- and downregulated genes in all three categories, this analysis shows that while many of the individual regulated genes may be different between *E. salsugineum* and *A. thaliana*, many of the cold-regulated functional classes of genes are the same. Interestingly, the Stress bin only showed an overrepresentation of up-regulated genes in *A. thaliana*. This is somewhat surprising, because one might expect the extremophile species to increase a larger set of stress-related genes. However, it should be kept in mind that this analysis only covered those *E. salsugineum* genes with an annotated ortholog in *A. thaliana*, corresponding to only about half of the cold-regulated genes in *E. salsugineum*. It is tempting to speculate that the other "unknown" half of the cold-regulated genes in the extremophile may contribute additional important stress tolerance functions that still remain to be discovered.

During cold acclimation, in addition to transcriptomic changes, also changes in primary and

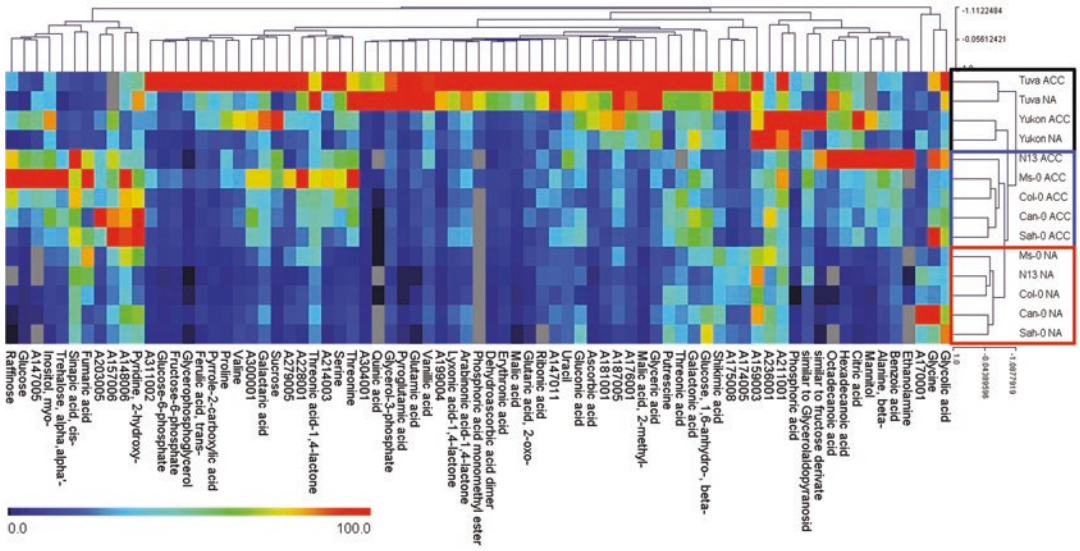

Fig. 5.4 Global overview of primary metabolite pool sizes before (NA) and after (ACC) 14 days of cold acclimation at 4 °C. Hierarchical clustering indicates that the metabolome of the two *E. salsugineum* accessions Tuva and Yukon (framed in black) was different from that of all *A. thaliana* accessions, both before and after cold acclimation. In *A. thaliana*, metabolite composition clearly separates non-acclimated (NA; framed in red) from acclimated plants (ACC; framed in blue). To compare all accessions, mass spectral responses were scaled to the highest sample, which was set to 100%

secondary metabolite composition have been observed in *A. thaliana* (Cook et al. 2004; Kaplan et al. 2004, 2007; Hannah et al. 2006; Guy et al. 2008; Maruyama et al. 2009; Schulz et al. 2015, 2016). In *E. salsugineum*, metabolomic studies have been performed on plants under salt stress (Lugan et al. 2010; Kazachkova et al. 2013; Lee et al. 2016), osmotic stress induced by polyethylene glycol (PEG, Lugan et al. 2010) and after 3 days of cold acclimation (Benina et al. 2013). Here we report gas chromatography-mass spectrometry (GC-MS) profiling of hydrophilic primary metabolites in the same *A. thaliana* and *E. salsugineum* accessions that were also used for the transcriptomic study reported above. Sample preparation, measurements and data analysis were performed exactly as described recently (Lee et al. 2016). A list of the content of all annotated analytes in all samples can be obtained from the corresponding author upon request.

We were able to detect 75 metabolites in our analysis and a hierarchical cluster analysis revealed three major sample clusters (Fig. 5.4). One cluster was formed by the two *E. salsugineum* accessions in both the non-acclimated and the cold-acclimated states. The other two clusters were made up of the *A. thaliana* accessions either before or after cold acclimation. In addition, after cold acclimation the accessions clustered following their relative freezing tolerance (compare Fig. 5.1). Such a clustering pattern of *A. thaliana* genotypes has been reported before (Hannah et al. 2006; Korn et al. 2010). The current results additionally indicate that *E. salsugineum* accessions have a different metabolic composition than *A. thaliana* accessions both under warm growth conditions (21 °C during the day; 18 °C during the night) and after 14 days of cold acclimation at a constant 4 °C.

To identify the main metabolites that are responsible for these metabolic phenotypes, we performed an independent components analysis (ICA, Daub et al. 2003) and extracted the top 10 metabolites underlying IC1 and IC2 (Fig. 5.5).

IC1 separates non-acclimated from cold-acclimated plants across both species and all accessions, while IC2 separates the two species, and within *E. salsugineum*, Tuva appears to be more divergent from *A. thaliana* than Yukon. A similar separation of the metabolic phenotypes of the *A. thaliana* accession Col-0 and the *E. salsugineum* accession Yukon was previously also found for hydroponically grown plants at different levels of salt stress (Lee et al. 2016), indicating that the differences in metabolite content between the two species are to a large extent independent of growth conditions. This is also in agreement with an earlier study comparing *A. thaliana* and *E. salsugineum* metabolomes in soil and agar grown plants under control and salt stress conditions (Kazachkova et al. 2013). It should also be noted that there is a large degree of cross tolerance between salt and freezing stress. In several plant species, salt stress induces higher freezing tolerance (Bender et al. 1992; Hincha 1994; Schmidt et al. 1986). On the other hand, in *E. salsugineum* cold induces higher salt tolerance, while this is not the case in *A. thaliana* (Shamustakimova et al. 2017).

The loadings analysis of IC1 (Fig. 5.5) indicates metabolites that distinguish non-acclimated from cold-acclimated plants. While the positive loadings of IC1 (i.e. those metabolites with higher content in non-acclimated than in cold-acclimated plants) comprise mainly metabolites that have not been identified yet, the negative loadings include the known cold-induced compatible solutes raffinose, glucose, mannitol and sucrose (see Guy et al. 2008 for a review). The data also show that the content of these metabolites after cold acclimation increases with increasing freezing tolerance of the accessions. This observation is in agreement with biochemical data for raffinose, glucose and sucrose in a larger collection of *A. thaliana* accessions (Zuther et al. 2012). The accumulation of raffinose in the cold was much weaker in *E. salsugineum* than in *A. thaliana*. Therefore, raffinose was also one of the top 10 positive loadings of IC2 (Fig. 5.5) underlying the separation of the two species in the ICA. Similarly, a higher accumulation of raffinose was also detected in *A. thaliana* under salt stress (Guevara et al. 2012; Kazachkova et al. 2013; Lee et al. 2016). Conversely, *E. salsugineum* shows a generally higher accumulation of proline under cold and salt stress conditions than *A. thaliana* (Guevara et al. 2012; Lee et al. 2012b, 2016; Kazachkova et al. 2013).

Interestingly, fumarate was also among the positive loadings of IC2, as it showed a higher content in *A. thaliana* than in *E. salsugineum* in the non-acclimated state and in addition a further accumulation in the cold only in *A. thaliana*. An accumulation of fumarate in *A. thaliana* has previously been shown over the first 8 h of cold treatment. This has been associated with photosynthetic acclimation to cold conditions. This hypothesis was based on data from a *FUM2* knock-out mutant with a disrupted cytosolic fumarase that no longer accumulated fumarate in the cold (Dyson et al. 2016). Apparently, *E. salsugineum* employs a different mechanism that does not involve fumarate accumulation to regulate photosynthetic acclimation to cold than *A. thaliana*. This conclusion is also supported by the fact that while downregulated genes common to both species were significantly overrepresented in the Photosynthesis bin, this was in addition only true for *E. salsugineum*-specific, but not for *A. thaliana*-specific genes (Fig. 5.3).

Contrary to expectation, however, we found a reduced expression of the *FUM2* (AT5G50950) gene mentioned above in the cold specifically in *A. thaliana* ($\log_2$ fold change between -1.266 and -3.495 as indicated by our microarray data), although fumarate levels were increased. Further research will be necessary to elucidate the differences in fumarate metabolism after growth under short (up to 8 h) and long (14 days) cold conditions and whether fumarate accumulation during cold acclimation in *A. thaliana* is necessary for increased freezing tolerance after acclimation.

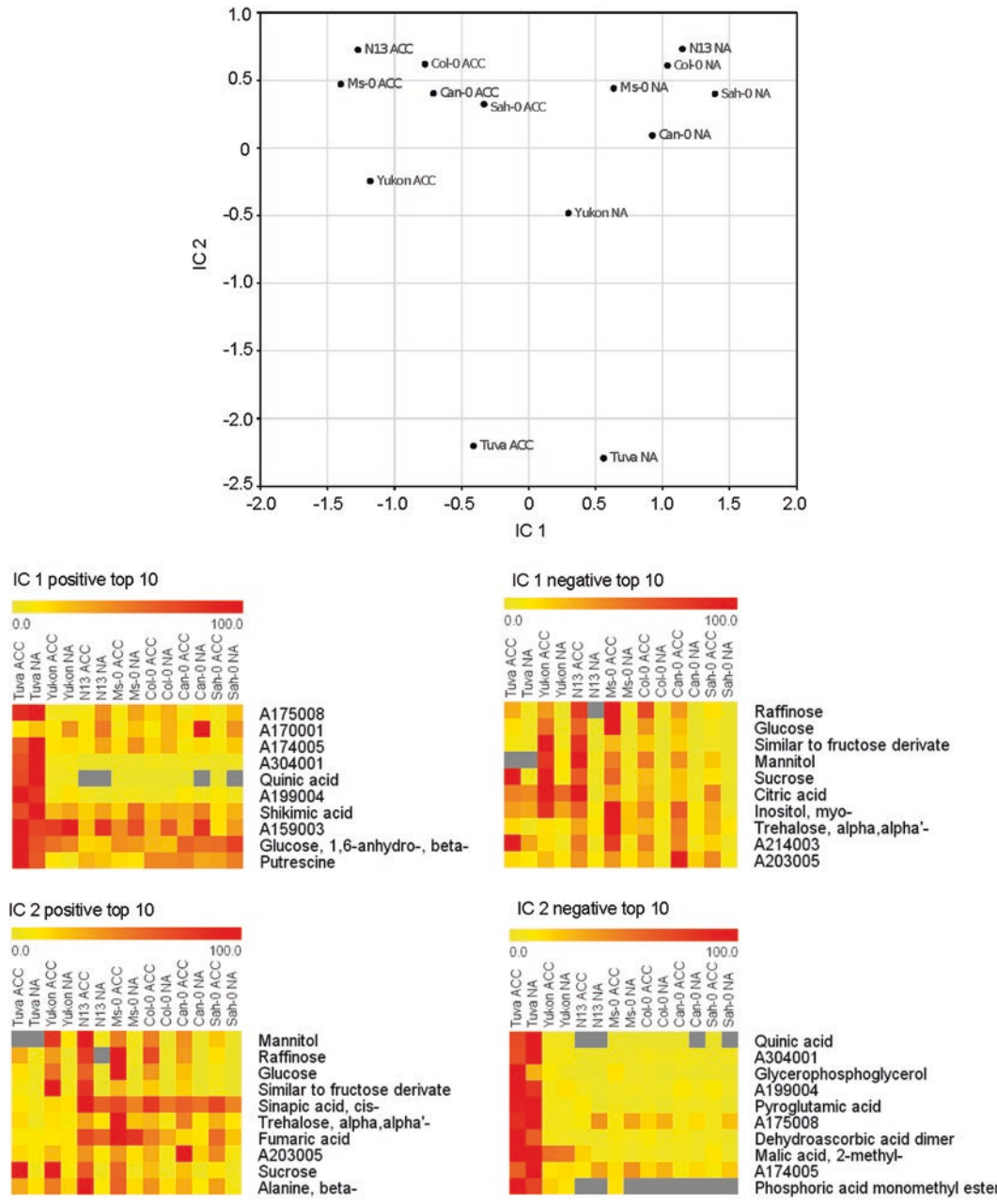

Fig. 5.5 Independent components analysis of pool size measurements of metabolites quantified by GC-MS. Independent components IC1 and IC2 separate cold-acclimated from non-acclimated plants (IC1) and both species (IC2). The top 10 metabolites from the positive and negative loadings of IC1 and IC2, respectively, are shown below the ICA. Red indicates high and yellow low metabolite content, expressed relative to the maximum content of each metabolite across all samples, set as 100%. Grey fields correspond to metabolite content below the detection limit

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6 Ice Nucleation Activity in Plants: The Distribution, Characterization, and Their Roles in Cold Hardiness Mechanisms

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Abstract

Control of freezing in plant tissues is a key issue in cold hardiness mechanisms. Yet freeze-regulation mechanisms remain mostly unexplored. Among them, ice nucleation activity (INA) is a primary factor involved in the initiation and regulation of freezing events in plant tissues, yet the details remain poorly understood. To address this, we developed a highly reproducible assay for determining plant tissue INA and noninvasive freeze visualization tools using MRI and infrared thermography. The results of visualization studies on plant freezing behaviors and INA survey of over 600 species tissues show that (1) freezing-sensitive plants tend to have low INA in their tissues (thus tend to transiently supercool), while wintering cold-hardy species have high INA in some specialized tissues; and (2) the high INA in cold-hardy tissues likely functions as a freezing sensor to initiate freezing at warm subzero temperatures at appropriate locations and timing, resulting in the induction of tissue-/species-specific freezing behaviors (e.g., extracellular freezing, extraorgan freezing) and the freezing order among tissues: from the primary freeze to the last tissue remaining unfrozen (likely INA level dependent). The spatiotemporal distributions of tissue INA, their characterization, and functional roles are detailed. INA assay principles, anti-nucleation activity (ANA), and freeze visualization tools are also described.

Keywords

Ice nucleation activity · Freezing behavior · Cold hardiness · Ice blocking barrier · Freeze regulating substances · Subzero temperature

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sensor · Freezing order · Supercooling · Anti-nucleation activity · MRI · Infrared thermography · Extracellular freezing · Freezing tolerance · Freezing avoidance · Water

Abbreviations

ANA	Anti-nucleation activity
DTA	Differential thermal analysis
INA	Ice nucleation activity
INT	Ice nucleation temperature
IR	Infrared
MRI	Magnetic resonance imaging
SEM	Scanning electron microscopy

6.1 Introduction

Freezing is a severe environmental stress for poikilothermic and immobile plants, whose major component is water. Higher plant tissues in general do not tolerate extreme desiccation except for orthodox seeds. Thus, retaining water within the tissues while avoiding lethal intracellular freezing is critical to survive freeze-thaw cycles. This physicochemically challenging task has been enabled in cold-hardy plants by the evolution of various methods to control tissue water behavior (phase and movement) under subzero temperatures. This resulted in various adaptive strategies of freezing (freezing behaviors) such as extracellular freezing (e.g., bark), deep supercooling (e.g., xylem ray parenchyma), and extra-organ freezing (e.g., cold-hardy azalea flower buds and conifer leaf buds) that are specific to both species and tissues (Sakai and Larcher 1987; Quamme 1995). More recently, the use of high-resolution MRI has revealed the occurrence of more diverse and complicated freezing behaviors (Ishikawa et al. 2016, 2018). Inherently, MRI can noninvasively visualize fine freezing behaviors by imaging the distribution of unfrozen water in plant organs (Price et al. 1997b). MRI successfully uncovered a unique set of freezing behaviors in wintering *Cornus florida* flower buds: only anthers and ovules remain unfrozen by supercooling, while the remaining flower bud tissues tolerate extracellular freezing (Fig. 6.1).

Yet, the underlying mechanisms that allow tissues to perform their specific intrinsic freezing behaviors including controlled management of ice nucleation and propagation, water flow or relocation, stabilization of supercooling, inhibition of ice crystal growth and/or recrystallization by antifreeze substances, and the occurrence of ice blocking barriers remain poorly understood (Ishikawa et al. 2009, 2016; Wisniewski et al. 2009). The mechanistic details, spatiotemporal distributions, and functional roles require clarification. This is in contrast with the accumulated research outcomes on alterations in membranes, lipids, sugars, and osmolytes and the expression of proteins, genes, and transcription factors as being correlated with freezing tolerance (tolerance of extracellular freezing as the freezing behavior) (Thomashow 1999). It also gives a striking contrast to the accumulated knowledge on ice management by antifreeze proteins in Arctic/Antarctic fish and cold-hardy insects (Fletcher et al. 2001; Scotter et al. 2006) as well as on ice nucleation activity/protein of ice-nucleating bacteria (Lindow 1983; Upper and Vali 1995; Warren 1995).

Ice nucleation is the primary event when plants encounter freezing temperatures and is thus a key factor in the initiation and regulation of tissue-/species-specific freezing behaviors and freezing processes. Ice nucleation of plants can be caused by extrinsic ice in the form of snow, frosts, frozen soil, or already frozen parts of the plant or by epiphytic ice-nucleating bacteria and by plant intrinsic ice nucleation activity (the ability of tissues to cause ice nucleation, hereafter referred to as INA). However, precise details of ice nucleation in plants as well as the contribution of plant intrinsic INA to ice nucleation and plant cold hardiness remain obscure (Wisniewski et al. 2009). More studies are required to characterize plant intrinsic INA as a cold hardiness mechanism, especially its precise spatial/temporal localization and distributions in

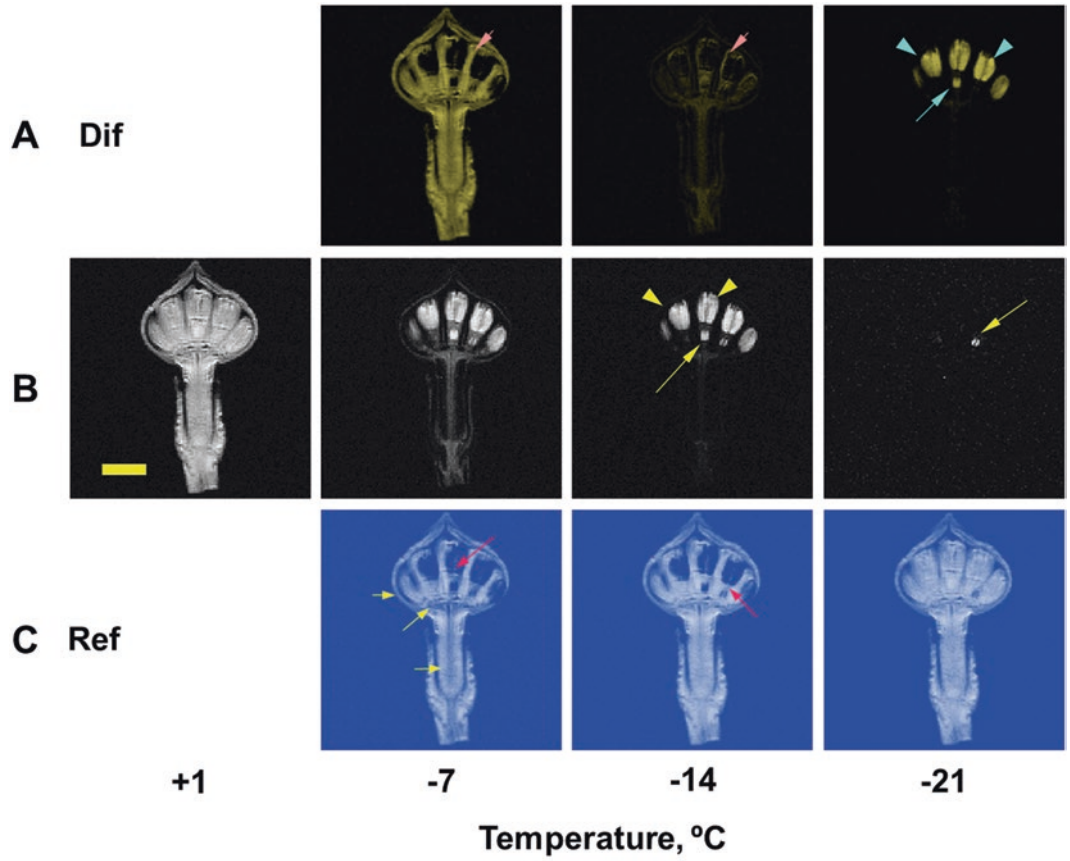

Fig. 6.1 Changes in the proton (^{1}H) density nuclear MRI images taken at subzero temperatures of a wintering *Cornus florida* flower bud. The center row (**b**) shows original raw MRI images of the same longitudinal slice taken at +1, −7, −14, and −21 °C (FOV: 16 × 16 mm). Intensity decreases in the raw images taken at neighboring designated temperatures are shown in the upper row (**a**: differential images). Intensity decreases in each raw image from the +1 °C image are shown in the bottom row (**c**: referential images). The bud was cooled at 5 °C/h and left at the designated temperature for 10 min before taking images. Instances of anthers (yellow arrow heads in **b**) and pairs of ovules (yellow-arrowed in **b**) remaining unfrozen at −14 and −21 °C are indicated. Many of the entire individual anthers and ovules remaining unfrozen at −14 °C abruptly disappeared (froze) at −21 °C, which is clearly displayed in the differential image of −14 and − 21 °C images (frozen anthers indicated by the blue arrow heads and frozen ovules by the blue arrows in **a**). Besides involucral bracts, receptacle and stem tissues (yellow-arrowed in **c**), calyxes, corollas (pink-arrowed in **a**), and pistil tissues (red-arrowed in **c**) excluding ovules likely underwent extracellular freezing (**c**). The bar indicates 3 mm. (Originally published in Ishikawa et al. 2016)

the plant kingdom and how it is regulated and what its functional roles are. This chapter addresses these questions and surveys the current status of plant INA research, which have not received proper attention in the cold hardiness community. Factors other than INA involved in freeze regulation and recent development in noninvasive freeze visualization tools are also reviewed.

6.2 Assay System for Determining Plant Tissue INA

To investigate plant tissue INA in detail, an accurate assay system is a prerequisite. Thus, we developed a highly reproducible INA assay system for small plant tissue specimens using a test

tube-based nucleation assay (Kishimoto et al. 2014a, b). Briefly, plant parts (e.g., bud scale, flower, or leaf primordium, 5–7 mm stem segment) are prepared by excising or detaching them after surface wiping the plant organs. An excised plant part is placed into pre-autoclaved Milli-Q water (fixed volume, either 0.5, 1, or 2 mL) in a TPX or glass test tube (usually 20–40 replicates for a sample) with a lid of transparent polyester film. The tubes are cooled in a stepwise manner in a precision-controlled bath (coolant: ~40% v/v ethylene glycol) from 0 to −20 °C in 1 °C decrements (cooling 1 °C took ~5 min). The tubes are maintained at each temperature for 20 min before counting the number of frozen tubes. INA is represented as the median of ice nucleation temperature (INT), at which 50% of the tubes freeze, since the median is marginally affected by erroneous outlying data points (if there are any) than the mean. Cumulative numbers of frozen tubes are often plotted against temperature to compare different sample INA (Fig. 6.2c). Using this system, the control tubes with water (without samples) show an average median INT of −19.2 °C (no test tubes ice-nucleated above −15 °C). In the presence of a sample in the tube, any shift of the median INT to temperatures above −15 °C is considered to derive from the sample INA.

Methods to determine INA and their theoretical basis were first developed to study cloud physics and precipitation mechanisms (Vali 1971, 1995). The freezing of water droplets containing aerosol particles was observed on cooled copper plates, and from this the number of INA (per unit volume) active above a designated temperature could be estimated (Vali 1971). Generally, ice nucleation increases with decreasing temperatures (temperature-dependent nucleation) and also with increasing exposure to a temperature (time-dependent or stochastic nucleation). An important finding was that ice nucleation in a small volume is dominated by temperature-dependent nucleation at all temperature ranges than time-dependent one whose effect begins to show up around −12 °C and increases at lower temperatures but remains persistently secondary (Vali and Stansbury 1966). The validity of all the current INA assay systems depends on the dominance of temperature-dependent nucleation. Larger water volume systems are increasingly prone to time-dependent nucleation, which may mask the temperature-dependent nucleation. Temperature-dependent nucleation dominancy similar to Vali's observation (Vali and Stansbury 1966) was observed in our INA assay systems employing 0.5–2 mL water volumes (Kishimoto et al. 2014b).

The droplet INA assay was effective in discovering ice-nucleating bacteria (Upper and Vali 1995). Attempts were made to apply or develop methods to determine plant INA (Ashworth and Davis 1984; Hirano et al. 1985; Gross et al. 1988). Their systems used 1–24 mL of water in the tubes, which had the median INT for the control (water only) without specimens around −11 to −12 °C, much higher than the median INT of our assay system. This may arise from the quality of water, too large water volume (more time-dependent nucleation effect), or the background INA level of the systems as discussed elsewhere (Kishimoto et al. 2014a, b). In our INA assay, to reduce background INA, all the equipment that are to be in contact with the Milli-Q water in the assay tubes are autoclaved at 121 °C for 20 min, including the tubes, lids, and water itself except for the specimens. Other ways of sterilization (gas or gamma rays) are not necessarily effective in reducing the background INA. The background INA level in an assay system can be checked by leaving the tubes with control water at −15 °C for several days (less than 5% of the tubes freeze in our system). All the sample handling processes are carried out in a laminar flow chamber, and clean tweezers are used to avoid contamination from airborne ice nuclei of microbial or any other origin. The use of transparent film for lidding the tubes and fiber optic illumination allows frozen tubes to be easily located without touching the tubes, since vibration of tubes at lower temperatures can often nucleate the tubes (Kishimoto et al. 2014b).

These things allowed our assay to obtain highly reproducible INA for a wide range of samples having both high and low INA (Fig. 6.2, Table 6.1) (Ueda et al. 2002; Sekozawa et al. 2002; Ishikawa 2014). A remaining issue is that tissue INA lower

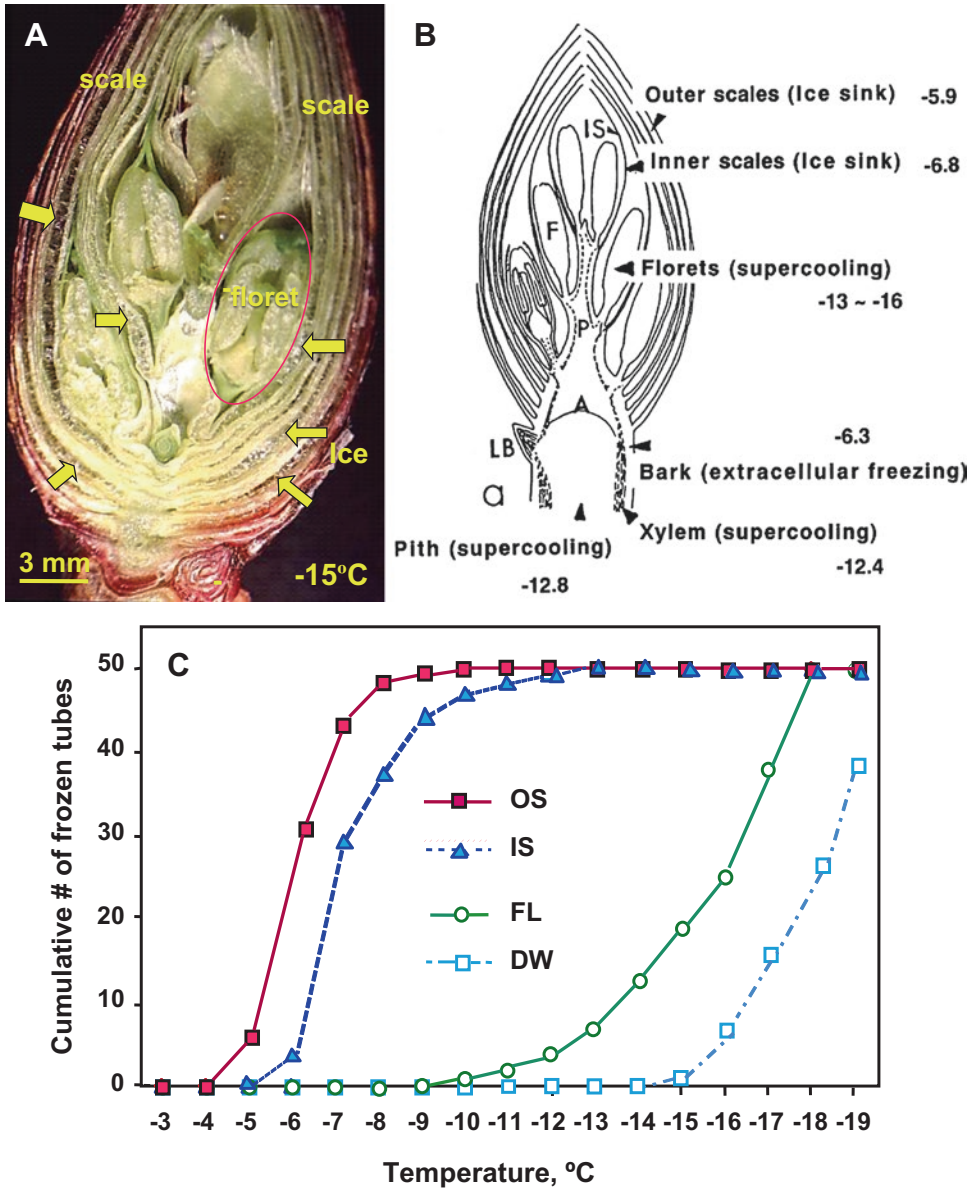

Fig. 6.2 A flower bud of *Rhododendron japonicum* viewed at −15 °C (**a**: ice crystals depicted by the arrows) and INA expressed as the median INT at which 50% of the test tubes froze determined with the test tube assay (**b**, **c**). One of the florets is marked with a red circle in (**a**). Each tissue name is followed by its intrinsic freezing behavior in the parenthesis and INA values (°C) in (**b**). (Originally published in Ishikawa 2014)

than −15 °C cannot be properly determined using this system as the control tubes without samples have median INT around −19 °C. Control tube freezing temperature can be lowered by reducing the water volume in the assay system. Yet, smaller water volumes restrict the tissue size that can be added. An alternative is to use homogenized sample suspension in droplets (Brush et al. 1994); however this may destroy INA responsible substances or structures and also allow the leakage of cellular contents that likely have anti-nucleating activity (ANA, the ability to suppress INA), which will be detailed later (Fig. 6.6). The smaller volume assay is more susceptible to the presence of

Table 6.1 Ice nucleation activity (INA) expressed as the median INT in various tissues of cold-hardy and cold-tender plants

Plant species/tissues or organs	INA (median INT), °C	
	Intact	AC/water changed
<Cold-tender plants>		
Oryza sativa	−12.5	−14.3
Flag leaves before heading		
Synsepalum dulcificum		
Leaves	−10.2	−13.2
Stems (current year)	−10	−10.7
Manihot glaziovii		
Stems (current year)	−11.2	−13.1
<Wintering cold-hardy plants>		
Phyllostachys bambusoides		
Leaves	−6.2	−13.5
Camellia japonica		
Stems (current year)	−5.9	−12.4
Morus bombycis	−6	−10.5
Stems (current year)		
Rosa rugosa	−5.6	−5.5
Stems (current year)		
Rhododendron japonicum		
Flower bud outer scales	−5.7	−5.5
Flower bud inner scales	−6.4	−6.6
Florets	−15	−17.3
Stems	−6.1	−12
Bark	−6.2	−13.3
Xylem	−12.3	−13.8
Pith	−12.4	−12.5
R. obtusum var. *kaempferi*		
Flower bud leaf-like scales	−5.7	−10.6
Flower bud outer scales	−6.4	−11.3
Florets	−15.3	−18.4
Stems	−6.2	−11.3

Originally published in Ishikawa (2014)
INA of intact tissues and autoclaved tissues (121 °C, 15 min) after replacing the supernatant with fresh sterilized water (AC/water changed) was determined using the test tube INA assay

ANA than the larger volume assay (for the same amount of tissue) since ANA substance concentration released from the samples into the assay water becomes higher.

The number of tubes required for significant INA determination depends upon the variance of tissue INA, but usually 20–50 tubes are used for a tissue sample (Kishimoto et al. 2014a, b). The sample amount in a tube can be altered for more qualitative analyses when required (Kishimoto et al. 2014b). In the test tube assay, detached/excised tissue samples are exposed to equal temperatures in the circulating bath. This allows the ability of each tissue to cause heterogeneous ice nucleation to be fairly compared (Kishimoto et al. 2014b). In DTA or thermography analyses of intact organs, the outer tissues are often exposed to lower temperatures than the inner tissues during the cooling process. Such a factor can be circumvented in the INA assays.

6.3 Localization of INA in Plant Tissues and Organs

6.3.1 Spatial Distribution of INA Within Plant Organs and Their Functional Roles

The developed INA assay was used for determining INA of various tissues in wintering flower buds (Fig. 6.2) and stems (Fig. 6.3). In *Rhododendron japonicum* flower buds, outer and inner bud scales showed high INA levels, while florets had low INA levels (close to the lower detection limit of the system) (Fig. 6.2b, c, Tables 6.1 and 6.2), irrespective of collection/cultivation sites of the buds (Ishikawa et al. 2015). This is in good agreement with the freezing behavior of the buds: bud scales freeze early and work as an ice sink in extraorgan freezing (Fig. 6.2a), while florets remain supercooled (Ishikawa and Sakai 1981; Ishikawa et al. 2015). It is likely that the high INA in bud scales functions as a freezing sensor, ensuring the primary freezing in bud scales at warmer subzero temperatures, which creates the vapor pressure difference ($P_{solution} > P_{ice}$) that drives the water migration from florets to bud scales and, consequently, the accumulation of ice crystals within the scales. The low INA in florets likely helps them remain unfrozen through stable deep supercooling. The INA distribution within flower buds (high in scales and low in florets) likely plays a crucial role in the

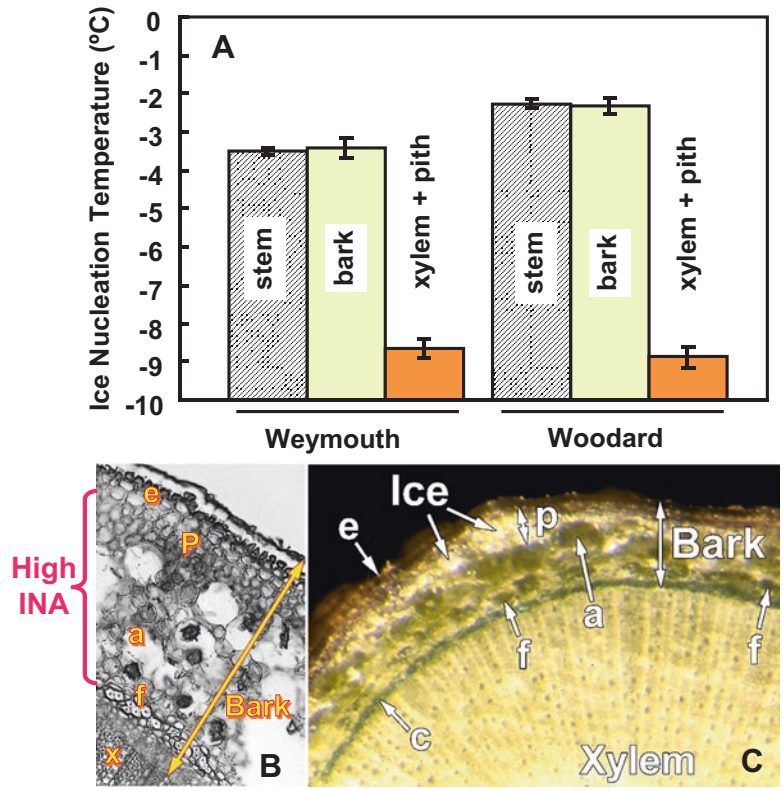

Fig. 6.3 INA of current-year blueberry stems and their component tissues (cv. Weymouth and Woodard) collected in December determined with the INA assay (**a**). Bark tissues were carefully separated from the xylem plus pith with a knife for INA assay. A cross section viewed at 25 °C of current-year blueberry stems (cv. Weymouth) collected in late December (**b**) and ice crystal localization in the corresponding stems cooled at −2 °C/h and observed at −10 °C (**c**). Ice crystals are shown with arrow heads. *e* epidermis, *c* cambium, *f* fiber, *a* aerenchyma, *p* cortical parenchyma. (Originally published in Kishimoto et al. 2014b)

initiation and subsequent process of extraorgan freezing (Ishikawa and Sakai 1985).

The distribution of INA in *R. japonicum* stem tissues was also determined (Table 6.1, Fig. 6.2b). Bark tissues had high INA (−6 °C), while the xylem and pith had much lower INA (−12 to −13 °C) (Ishikawa et al. 2015). This corroborates the intrinsic freezing behavior of the tissues: high INA in the bark which spontaneously freezes at warm temperature to undergo extracellular freezing while low INA in the pith which remains unfrozen by deep supercooling as visualized in MRI studies (Price et al. 1997a).

Tissue localization of stem INA was also studied in two blueberry species, which had much higher INA (median INT of −2 to −3 °C) in the stem. The high INA was localized in the bark, while the xylem had much lower INA (Fig. 6.3). INA distribution corroborates the freezing behaviors of the tissues in a similar manner as *R. japonicum* stems (Kishimoto et al. 2014b). The high INA in the bark may also help extracellular ice formation in specific sites (cortex working as an ice sink) by allowing earlier initiation of freezing (Fig. 6.3c). It may also help retain the ice crystals by promoting recrystallization in the cortex as ice management to avoid freeze desiccation of the tissues, which is currently only hypothetical.

Lengthwise, INA was evenly distributed along the current-year stem axis (Kishimoto et al. 2014b). This implies that the stem could spontaneously initiate freezing at any lengthwise position. This was confirmed with infrared (IR) thermography observation (Yamazaki and Ishikawa 2010; Yamazaki et al. 2011). Differential thermography images revealed that freezing in blueberry stems initiated in the bark concurrently at various longitudinal positions of the stem and propagated slowly within the bark, which was later transmitted from the bark to the xylem and rapidly spread to the entire stem through the xylem (Yamazaki et al. 2011). This freezing process (the order of tissue freezing: the bark first and the xylem later) agreed well with the INA distribution in the stem (Fig. 6.3).

Table 6.2 Effect of sample amount, grinding and autoclaving on the ice nucleation activity (INA) in bud scales of wintering *Rhododendron japonicum* flower buds

	Ice nucleation activity (median INT), °C		
R. japonicum Tochigi (Nov 22)	Intact	AC	AC/WC
IS × 1	−6.6 ± 1.0	−7.8 ± 1.2	
IS × 4	−5.9 ± 0.6	−6.6 ± 0.7	
OS × 1	−5.6 ± 0.4	−7.7 ± 0.5	
OS × 4	−5.2 ± 0.4	−6.6 ± 0.5	
OS powder × 1	−5.5 ± 0.4	−7.4 ± 0.2	−5.8 ± 0.4
OS powder × 4	−5.3 ± 0.5	−6.9 ± 0.5	−5.4 ± 0.3
OS powder × 16			−5.3 ± 0.2
OS powder × 32			−4.7 ± 0.2

Tubes with 2 mL of autoclaved Milli-Q water plus intact specimens were used for INA assay (Intact). After the assay, the tubes with samples were autoclaved at 121 °C for 15 min and directly used for another INA determination (AC) or after replacing the supernatant with fresh cold sterilized water and used for INA assay (AC/WC). A single or four pieces of outer scale (OS), inner scale (IS), or the equivalent amount of homogenate powder was added to each test tube for the INA assay unless otherwise specified. The data are presented in the form of median INT ± SD, so that the variance of INT can be seen (two-thirds of the INT values are within the SD range). Originally published in Ishikawa (2014)

The order of tissue freezing differs considerably in *Forsythia* stems, where freezing initiated in the pith, transmitted to the xylem, and finally to the bark as revealed by thermography (Ishikawa 2016). Correspondingly, *Forsythia* stems had high INA (−2 to −3 °C) in the pith and low INA in the bark and xylem (−8 to −10 °C). The order of tissue freezing in an organ seems to be specific to species and corresponds well with the distribution of tissue INA in the organ.

These studies have downplayed the generally held view that freezing in trees initiates in the xylem (Sakai and Larcher 1987). This theory is attributed to the large volume of water in xylem vessels (Franks 1985), and based on the finding by Kitaura (1967), that freezing was propagated through the xylem in mulberry stems in spring. Yet, they did not determine where exactly the freezing initiated.

6.3.2 Subcellular Localization of Bark Tissue INA and Their Functional Roles

Subcellular localization of the INA in blueberry bark tissues was studied by homogenization and fractionation using differential centrifugation (Kishimoto et al. 2014b). The highest INA (expressed as median INT) was detected in the homogenate (−6.5 °C) and the cell wall fraction (−6.6 °C). Much lower INA was detected in nuclear rich (−11.4 °C), mitochondria and plastid rich (−16.3 °C), microsomes (−10.8 °C), and soluble fractions (−13.0 °C). Microscopically, the cell wall fraction comprised cell walls and structural components such as fibers in the bark. Rinsing the cell wall fraction with 0.5–1 M NaCl to remove ionically bound substances did not affect the high INA in this fraction.

The results suggest that the INA of blueberry bark tissues is localized and tightly bound in the cell walls and/or intercellular structural components. Rationally, the presence of high INA in the cell walls (presumably on their outer surface) and/or neighboring structural components facilitates ice formation outside the cells or intercellular space in the bark (Fig. 6.3c) at warm subzero temperatures. This allows the cells to undergo extracellular freezing due to the vapor pressure difference between the extracellular ice and intracellular cell sap ($P_{solution} > P_{ice}$). The lack of efficient ice nucleators plus the presence of ANA (detailed later) inside the cells likely contributes to retaining the cells in supercooled state until the completion of cell water migration to extracellular ice. The occurrence of INA in a specific tissue and a specific cellular component also implies that the INA in the blueberry stem is more likely of intrinsic origin rather than of microbial origin.

If the INA is of microbial origin, a strict control of its localization by the host (blueberry stems) would be required, which is unlikely.

In summary, the distribution of tissue INA in an organ corroborates freezing behaviors of the tissues, the order of tissue freezing (from the primary freeze to the last tissue remaining unfrozen), and preferential localization of ice crystals in the tissues. High tissue INA likely works as a freezing sensor, ensuring freeze initiation at warmer subzero temperatures of the tissue since extrinsic ice does not necessarily nucleate plants providing the epidermis cuticles are intact (Hacker and Neuner 2008).

6.4 Seasonal Alterations of Tissue INA

For better understanding of plant tissue INA, its development and seasonal changes were followed. In *R. japonicum*, flower bud morphogenesis comes to an end in late August. INA levels of outer and inner bud scale increased from late August to late October (Fig. 6.4) just in time for the first autumnal frosts when a subzero temperature sensor function is required to survive the first freeze. Outer and inner scales retained high INA levels during the winter, but their levels gradually declined in the spring when flower buds resumed growth (the bud scales still attached). INA in florets stayed at low levels from August until the end of winter, but it went up in the spring as floret dry weight rapidly increased.

Seasonal changes in stem (7.5 mm segments) INA of two blueberry cultivars were also studied (Kishimoto et al. 2014a). Newly developed shoots in early May showed low levels of stem INA but rapidly increased INA levels until July when the stems became mechanically hard and seemingly matured. From July to November, the stem INA increased steadily and gradually, having the maximum INT (−2.2 and −1.7 °C in Weymouth and Woodard, respectively) in October or early November, just before the first autumnal frost. As they experienced recurrent frosts, stem INA decreased with oscillations and reached the lowest level in February but went up again in March. INA of previous year stems showed similar chronological changes to that of current-year stems.

In both cases (flower bud scales and stem bark), tissue INA increased to the highest level, just in time for the first autumnal frosts when a subzero temperature sensor function is required to survive the first freeze. It appears that some substances responsible for the INA are actively synthesized in these tissues. It is known that the first autumnal frosts accelerate cold acclimation (Kuroda et al. 1990) and surviving the first frosts is likely important.

6.5 Distribution of INA in Plant Kingdom

The INA of various tissues of more than 600 species has been surveyed. Freezing-sensitive tropical and subtropical plants tend to have low INA levels in their tissues (Table 6.1) (Ishikawa 2014). Conversely, cold-hardy plants tend to have high INA in some specific tissues as detailed earlier (Table 6.1, Figs. 6.2, 6.3, and 6.4). The lack of INA and the presence of ANA (detailed later) in freezing-sensitive plants allow them to remain unfrozen by transient supercooling. This could be a primitive strategy to survive short-term exposure to subzero temperatures. However, when exposed to lower subzero temperatures (−8 to −12 °C), transiently supercooled tissues tend to cause lethal intracellular freezing due to the high ice crystal growth rates in water supercooled to −8 to −12 °C (Fig. 6.5) (Pruppacher 1967; Tsushima 2015). Conversely, cold-hardy plants seem to have evolved high INA in specialized tissues (extracellular spaces), where the high INA functions as a freezing sensor and induces freezing at warmer subzero temperatures (−1 to −6 °C). This temperature range has much slower ice crystal growth rates (Fig. 6.5) and likely allows enough time for migration of cellular water to growing extracellular ice crystals.

The INA survey revealed an extremely high INA in blueberry stems (cv. Woodard). This INA was localized in the cell wall fraction of bark tissues as detailed above. The highest INA (−1 to

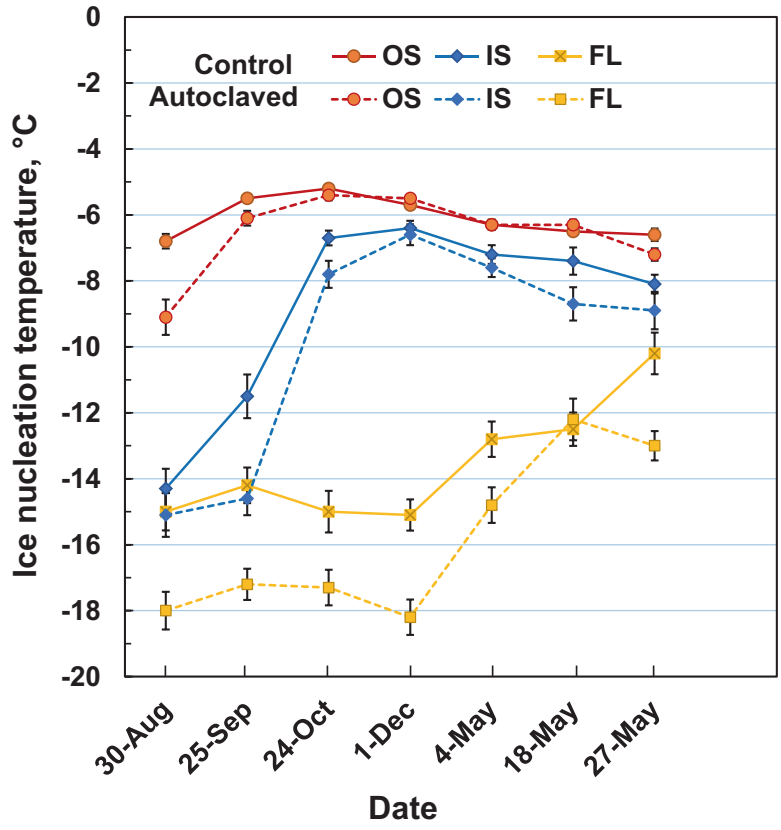

Fig. 6.4 Seasonal changes in the INA of intact flower bud parts of *R. japonicum* (control) and INA after autoclaving and replacement of water (autoclaved) showing the stability of INA at 121 °C for 15 min. INA was determined as the median INT using the test tube assay. *OS* outer scale, *IS* inner scale, *FL* floret. The data are mean ± SE ($n = 3$). (Originally published in Ishikawa et al. 2015)

−2 °C) was consistently observed with 7.5 mm stem segments in October or early November. This may be one of the highest INA of biological and chemical origins ever reported, much higher than the INA of ice-nucleating bacteria (−2.5 to −3.0 °C) and AgI (−3.1 °C). Currently, we are trying to identify the substance responsible for blueberry bark INA.

6.6 Characterization of Plant Tissue INA

Autoclaving experiments (121 °C for 15 min) indicate that two distinct types of INA exist within the *R. japonicum* plant: heat-resistant type in the bud scales and heat-labile type in the bark (Tables 6.1 and 6.2, Fig. 6.6). In *R. obtusum*, bud scale INA is also heat-labile as well as stem INA (Table 6.1) (Ishikawa 2014). INA in blueberry stems is also heat-labile. These two types of INA could be related as implicated by the temporal increases in the autoclaving resistance of INA in *R. japonicum* flower bud scales (Fig. 6.4). Outer scale INA was moderately sensitive to autoclaving (2 °C decrease) in late August but became resistant to autoclaving in late September. Conversely, inner scale INA was sensitive to autoclaving from late August until late October (1–3 °C decrease) but became resistant in early December.

The INA in the bud scales is not derived from the macrostructures such as trichomes as it is unaffected by grinding to fine powder (Table 6.2) and is likely attributed to some substances that are resistant to autoclaving (121 °C for 15 min). This is in contrast to the heat-labile property (inactivated at 80 °C) of the known microbial INA (Ashworth and Kieft 1995; Hirano and Upper 1995). These properties indicate that the bud scale INA of wintering *R. japonicum* flower

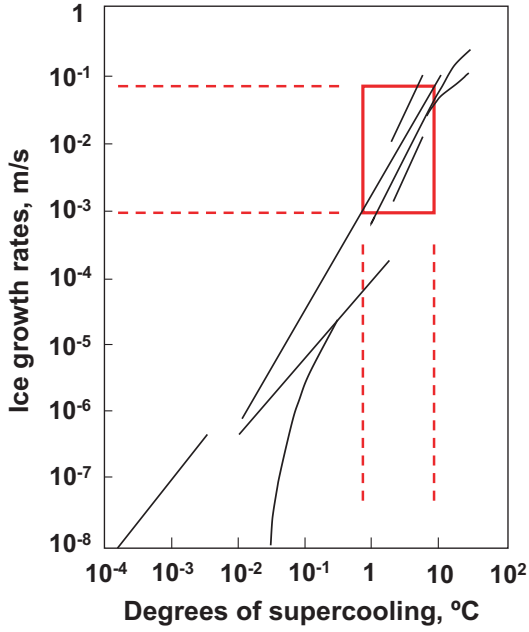

Fig. 6.5 Relationship between ice crystal growth rates and the extent of supercooling of water. Each of the multiple curves represents determination by different researchers including Pruppacher (1967). The red box and doted lines indicate ice growth rates at −1 and −10 °C, where the latter is approximately 100 times faster than the former. (This is adapted from Tsushima 2015)

buds is likely of intrinsic origin rather than microbial origin.

Ice nucleation activity of bacterial origin has been characterized in detail (reviewed in Hirano and Upper 1995; Upper and Vali 1995). High INA has also been found in fungi (some *Fusarium* species) (reviewed in Ashworth and Kieft 1995). The INA of these organisms was considered to be proteinaceous (reviewed in Ashworth and Kieft 1995; Fall and Wolber 1995), and the gene responsible for the ice nucleation-active protein was identified in ice-nucleating bacteria based on the mutant analysis (Warren 1995). In contrast, attempts to isolate the substances responsible for plant intrinsic INA have been unsuccessful as they often resulted in a loss of activity or because solubilization proved difficult (Pearce 2001). This allowed some to believe that plant ice nucleators are mostly of epiphytic microbial origin. However, woody plants have INA with different properties from those of microbial origin (Ashworth and Kieft 1995). Previous attempts to identify plant intrinsic ice nucleators ended up with only partial characterization (e.g., Ashworth and Davis 1984; Gross et al. 1988; Brush et al. 1994), and the substances responsible for the INA have not been successfully identified (Wisniewski et al. 2009).

Other than the two types of INA (differing in autoclave resistance) described earlier, three more types of plant-derived INA have been observed in our INA survey. Identification of these INA responsible compounds and their action mechanisms are currently under investigation in our laboratory.

6.7 Anti-nucleation Activity (ANA) in Plant Tissues

The autoclaving-induced INA decreases and the subsequent INA increases by water replacement typically observed in *R. japonicum* bud scales (Table 6.2, Fig. 6.6) can be interpreted as follows. The supernatant (leachate from the autoclaved scales) likely has anti-nucleation activity (ANA), defined as the ability to suppress the INA of a substance or tissues (Ishikawa et al. 2009), which can be removed by replacing the supernatant with fresh sterilized water. The solute concentrations of the autoclaved scale supernatant were less than 10 mOsm/kg. The detected ANA is unlikely attributable to the colligative effects of the solutes (freezing-point depression). Similar ANA has been observed in the autoclaved leachate from most of the plant tissues surveyed (over 600 species), irrespective of their origins, woody or herbaceous, and levels of cold hardiness (Ishikawa 2014). The leachate from some tropical plant tissues showed even higher ANA levels than cold-hardy ones, and this is likely a general phenomenon (Ishikawa 2014). ANA was also observed in the leachate from the tissues killed by other methods such as liquid nitrogen immersion. This implies that free ANA is primarily located inside the cells.

Initially, high ANA that suppresses the bacterial INA was found in the extracts of deep-supercooling type of tissues such as cold-hardy

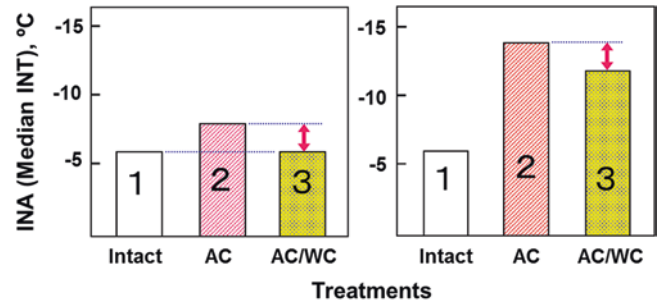

Fig. 6.6 Typical responses of intact plant INA (bar 1) of heat-stable type (**a**) and heat-labile type (**b**) to autoclaving at 121 °C for 15 min (AC, bar 2) and to subsequent replacing the supernatant leachate with fresh sterilized water (AC/WC, bar 3). Intermediate INA types showing heat sensitivity between these two extreme types have been also observed (Ishikawa 2014). Anti-nucleation activity (ANA) indicated by the red arrows are generally observed in all the INA types. (Originally published in Ishikawa 2014)

palm leaves (*Trachycarpus fortunei*) and xylem ray parenchyma of woody species (Larcher et al. 1991; Kasuga et al. 2008; Ishikawa et al. 2009). These ANA are ascribed to amphiphilic flavonoid glucosides, active at less than 10 mM concentrations. The high ANA found in *T. fortunei* leaf extracts suppressed the median INT of ice-nucleating bacteria from −5 to −14 °C at less than 1 mM concentrations, and the ANA was assigned to luteolin-7-glucoside and related compounds (Ishikawa et al. 2009; Ishikawa 2016). As described earlier, however, ANA was found to occur not only in deep-supercooling tissues but also in the extract of all types of tissues including extracellularly frozen tissues and freezing-sensitive tropical plants (Ishikawa 2014, 2016). The extract from cold-hardy *R. japonicum* florets that have high deep supercooling capability did not necessarily show high ANA levels. Flavonoids and polyphenols are known to occur throughout the year in most higher plant tissues including tropical plants. These findings imply that ANA is correlated with plant tissues in general (including freeze-sensitive and excluding cold-hardy tissues with high INA) having low INA levels and tending to supercool in response to subzero temperatures (Table 6.1). These soluble flavonoid glucosides are likely localized within the cells and help them remain temporarily unfrozen as a simple strategy to avoid lethal intracellular freezing upon transient exposure to subzero temperatures in all types of plants (from tropical to cold-hardy plants) irrespective of seasons (winter or during growing seasons). This mechanism may hold true with extracellularly frozen tissues with high INA. High INA most likely localized in the extracellular spaces as described earlier (Kishimoto et al. 2014b), while ANA responsible compounds are likely localized intracellularly. Thus, they would not interfere with each other: the INA promotes ice formation at warm subzero temperatures, while the ANA help the cells remain unfrozen during the process of extracellular freezing. Yet, when the cells are ruptured by autoclaving, the ANA responsible compounds leak out to extracellular spaces where they likely suppress the INA. These possibilities and hypothesis remain to be further investigated.

6.8 Other Factors and Mechanisms Involved in Freezing Behaviors

Other than the specific localization of tissue INA, some other freeze-regulating mechanisms are conceptually required to establish complex freezing behaviors where some tissues freeze early and adjacent tissues remain stably unfrozen by supercooling as typically seen in *Rhododendron* and *Cornus* flower buds (Figs. 6.1 and 6.2). These include the following: (1) occurrence of substances that stabilize the supercooling in tissues that remain unfrozen (e.g., florets or anthers); (2) the presence of ice blocking barriers that prevent ice intrusion from the already frozen tissues into

the supercooled tissues (presumably localized on the surface of supercooled tissues and connecting tissues such as pedicels or filaments); and (3) some of the ice blocking barriers should stop or restrict the vapor pressure difference ($P_{solution} > P_{ice}$) dependent water migration or relocation from the supercooled tissues to the frozen tissues (e.g., the barriers on the floret surface), while other ice blocking barriers are permeable to water (either in the form of vapor or liquid) and allow slow migration of water (e.g., the barriers in floret pedicels of *R. japonicum*).

In *R. japonicum* flower buds where the entire florets remain supercooled (−20 °C or lower), the florets have extremely low levels of INA (Fig. 6.2 and Table 6.1) while cell sap osmolarity of no greater than 1.2 Osm/kg (Ishikawa et al. 2015). The supercooling capability cannot be attributed to the colligative effect of cell sap osmolarity. When *R. japonicum* flower buds are slowly cooled, they undergo extraorgan freezing where some of the water in supercooled florets gradually migrates through the pedicels to frozen bud scales, resulting in further enhancement of the floret supercooling capability (Ishikawa et al. 2015). This extraorgan freezing process can be established by the coordination of the above-described three mechanisms in addition to the high INA in bud scales and low INA in florets. When the buds are thawed, the increased water in the scales slowly goes back to the dehydrated florets likely through the pedicels. This water relocation is most likely mediated by the higher osmolarity in florets as the driving force. We found that the extract of *R. japonicum* florets has low levels of ANA as described earlier, which implies that compounds other than flavonoids are responsible for the high supercooling capability in florets. Identity of these factors and mechanisms remains to be studied.

6.9 Recent Advances in Freeze Visualization Tools

Freezing events inside complex thick organs like woody plant winter buds are not readily visible and difficult to analyze. This is a major obstacle to investigating fine details (dynamics, tissue interactions in vivo) of freezing behaviors, their diversity, and freeze-regulation mechanisms. Thus, the development of noninvasive freeze visualization tools is crucial and would corroborate localization of freeze-regulating activities/compounds, or vice versa. It would also complement conventional methods used for studying plant freezing behaviors such as differential thermal analyses (DTA), microscopic observation, and Cryo-SEM, which have problems of either being unable to spatially locate the freezing or supercooling events in the sample or being destructive (Ishikawa et al. 2016). Inherently noninvasive visualization tools such as infrared (IR) thermography and MRI would simultaneously allow nondestructive, continuous, and spatially specific analyses done on the same sample during a freeze-thaw cycle.

6.9.1 IR Thermography

IR thermography detects the latent heat flow released from the surface of tissues being frozen and can visualize rapid freezing process including ice nucleation and propagation in intact plants (Wisniewski et al. 1997, 2009; Ball et al. 2002). Recent improvements in thermography sensitivity and image analyses have allowed successful imaging of detailed freezing processes. Subtracting a selected reference image from the original raw images helps reduce background noise (referred to as IDTA or referential imaging), resulting in the improved image quality and analyses (Hacker and Neuner 2008; Yamazaki and Ishikawa 2010). Using this technique, Neuner's group has extensively explored tissue level freezing behaviors (strategies) in alpine plants during the summer growing period as the temperature frequently drops to minus in alpine zones where avoiding frost injuries is a vital survival strategy (Neuner 2014). As yet, IR thermography is unable to visualize tissues that remain unfrozen. The method has also difficulty in accurately detecting freezing events localized in inner tissues (away from the surface) unless otherwise dissecting the sample and losing the benefit of

being nondestructive. Neuner and his coworkers attempted to shoot partly incised samples and successfully visualized freezing events occurring in inner alpine and woody plant tissues (Neuner et al. 2010; Kuprian et al. 2014).

More recently, we have further improved the sensitivity of thermography images by employing differential imaging, where the neighboring images are subtracted to extract image differences (Yamazaki et al. 2011). This technique can pick up the freezing front of not only rapid and immense events but also very faint heat flow from minor or slow freezing events and allows more complicated freezing behaviors in wintering plant tissues successfully visualized (Yamazaki et al. unpublished).

6.9.2 MRI

MRI (magnetic resonance imaging) can noninvasively probe the distribution of water in living organisms and has been used for visualizing diverse physiological processes in medicine, animals, and plants (Callaghan 1991; Gupta et al. 2014;). With high-resolution MRI, resolutions of 20–100 μm can be obtained, sufficient for tissue or organ level studies. It is, however, difficult to detect rapid phenomena while maintaining high resolution. In theory, it can noninvasively detect fine distributions of unfrozen water inside complex plant organs under freezing temperatures, while the signal from frozen water becomes undetectable as the T_2 relaxation time becomes extremely short (Price et al. 1997b). With such a contrast mechanism, MRI has successfully visualized typical freezing behaviors (e.g., extraorgan freezing) in wintering plant organs (Price et al. 1997a; Ishikawa et al. 1997; Ide et al. 1998). A theoretical background for interpreting MRI images taken at differing subzero temperatures was also considered (Price et al. 1997b).

MRI has turned out to be extremely useful for exploring novel freezing behaviors in complex plant organs and also elucidating the localization of ice blocking barriers which blocks the ice intrusion from the frozen tissues but is more or less permeable to liquid water or vapor as typically shown in Fig. 6.1 (Ishikawa et al. 2016). In addition, differential MRI images (subtraction of MRI images taken at two different temperatures: Fig. 6.1a) can spatially locate the tissues that have frozen or dehydrated between the designated temperatures. Referential MRI images (subtraction of the MRI image at each subzero temperature from the +1 °C image) indicate the distribution of tissues that had cumulatively frozen by the designated temperature (Fig. 6.1c). These techniques can visualize the distribution of frozen tissues in addition to unfrozen tissues, which also allows the boundary (ice blocking barrier) between frozen and unfrozen tissues to be clearly located. This makes the MRI more powerful for analyzing freezing behaviors and underlying mechanisms. More recently, improvement of MRI (higher magnetic strength and analytical tools) has allowed higher-resolution imaging, 3D imaging (Fig. 6.7), and even semi-real-time analyses. MRI can also probe the state of water and flow/diffusion of water in tissues exposed to biotic and abiotic stresses by employing different contrast mechanisms (Köckenberger 2001; Borisjuk et al. 2012; Dean et al. 2014; Fukuda et al. 2015). MRI successfully imaged seasonal alterations in xylem sap flow rates in a tree trunk in situ (Nagata et al. 2016).

"Freeze or not to freeze (remain unfrozen or prevent the tissue from freezing)" is a physicochemical event but also an important physiological function/process of plant tissues exposed to freezing temperatures. This function/process in the same sample can be imaged in consecutive and inherently noninvasive manners using MRI and IR thermography. These visualization tools and the conventional methods (DTA, optical microscopy, Cryo-SEM) provide complementary information. The combination of imaging, conventional tools, and freeze-regulation activity analyses is promising for exploring the diversity and mechanisms in freezing behaviors in complex organs. Currently, a major problem in utilizing high-resolution MRI may be the limitation in machine time (and expense) available for plant research.

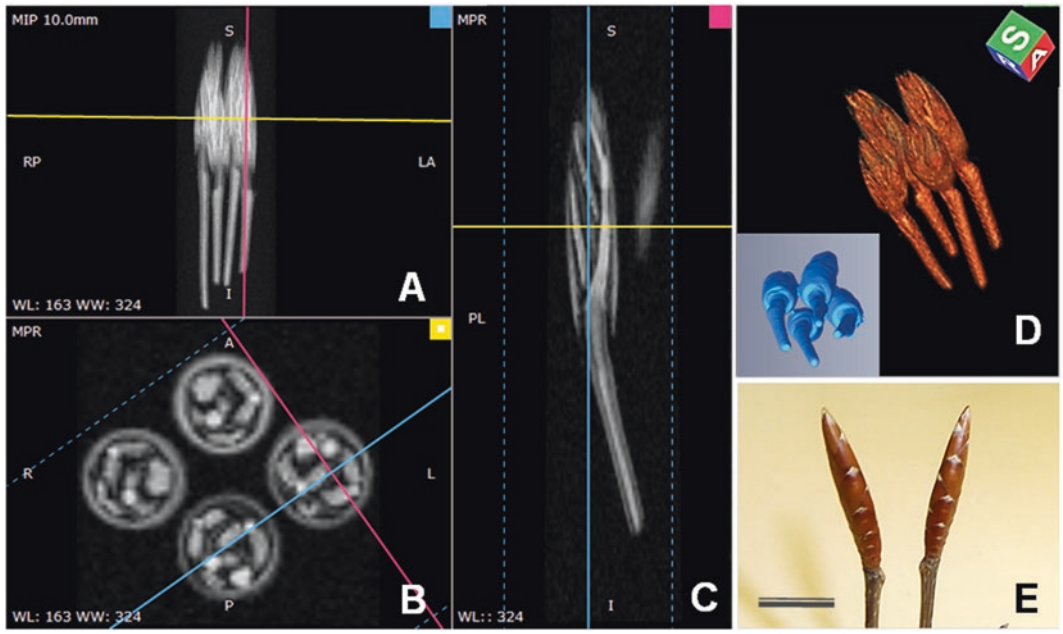

Fig. 6.7 3D MRI analyses of wintering terminal leaf buds of *Fagus crenata* (four buds wrapped and bundled using parafilm). The image analysis software allows visualization of slices in arbitrary directions (**a**–**c**) and the 3D images of different tissues (**d**). The horizontal (transverse) slice (**b**) is located at the yellow line in the vertical slice (**c**), which, in turn, corresponds to the pink line in (**b**). Vertical slices between the blue dotted lines (perpendicular to both pink and yellow line planes) are merged to show a panoramic slice image (**a**). 3D images of both the surface (blue inset) and interior tissues (brownish) can be constructed from the acquired 3D MRI dataset (**d**). MRI images show the bud inner structure: how primordial leaves and stems are folded and packed in the buds (**a**–**d**). The bar indicates 1 cm in an optical photo of *Fagus* leaf buds covered with numerous bud scales (**e**)

6.10 Concluding Remarks

Water in the ice state has much lower vapor pressure (P_{ice}) than cell sap or xylem sap in the liquid state ($P_{solution}$) at a given subzero temperature. This vapor pressure difference ($P_{solution} > P_{ice}$) generates the driving force for water migration and relocation, which could occur at the cellular, tissue, or organ levels, within the plant across the tissues/organs and between the plant and extraneous ice/snow. Cold-hardy plants seem to have evolved various mechanisms to control water behaviors (phase and migration). This results in various strategies of freeze survival such as extracellular freezing, extra-tissue freezing, extraorgan freezing, and deep supercooling, while freeze-thaw-associated water behaviors often cause unfavorable conditions such as xylem embolism (Venturas et al. 2017), excess tissue/organ desiccation, and trunk disruption (Sakai and Larcher 1987). Depending upon the extent, these could be recovered by appropriate mechanisms (e.g., xylem refilling) or otherwise become fatal.

Among the mechanisms involved in tissue water behavior regulation, the control of ice nucleation in appropriate places and timing seems to be vital as it is the primary event and creates the major vapor pressure difference ($P_{solution} > P_{ice}$). The presence and localization of ice blocking barriers that stop ice intrusion (freeze propagation) and simultaneously allow or restrict the movement of water (liquid or vapor) may be another important factor. This chapter summarized the present knowledge on plant tissue INA. INA and ice blocking barriers could be crucial in hydraulics or water management not only in organs but also in the whole plant under freezing temperatures, which is yet to be elucidated.

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7 Investigating Freezing Patterns in Plants Using Infrared Thermography

David P. Livingston III

Abstract

Since the discovery of infrared radiation in 1800, the improvement of technology to detect and image infrared (IR) has led to numerous breakthroughs in several scientific fields of study. The principle that heat is released when water freezes and the ability to image this release of heat using IR thermography (IRT) has allowed an unprecedented understanding of freezing in plants. Since the first published report of the use of IRT to study freezing in plants, numerous informative discoveries have been reported. Examples include barriers to freezing, specific sites of ice nucleation, direction and speed of ice propagation, specific structures that supercool, and temperatures at which they finally freeze. These and other observations underscore the significance of this important technology on plant research.

Keywords

Infrared thermography · Plants · Freezing · Latent heat · Ice nucleation · Ice propagation · Barrier · Supercool · Thermocouple

Abbreviations

IRT	Infrared thermography
MRI	Magnetic resonance imaging
NMR	Nuclear magnetic resonance

7.1 History

Infrared radiation was discovered by the astronomer, William Herschel, in 1800 when he duplicated Isaac Newton's experiments separating sunlight into its component wavelengths using a prism. He discovered that the different wavelengths raised the temperature of a thermometer by different amounts. Interestingly, he noticed radiation beyond the red portion of the spectrum that was not visible and raised the temperature by a greater amount than any visible wavelength. He called this radiation "calorific rays" and demonstrated that it obeyed the same laws as visible light (Gaussorgues 1994). By 1830 the first detectors were developed to measure infrared radiation using the principle that heat effects the conduction of electricity, as in a thermocouple. The bolometer, invented by the American astronomer Samuel Langley in 1878, was the first significant improvement in infrared (IR) detection. The bolometer was similar to existing thermal detectors in that it was based on the principle of the temperature dependence of electrical

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resistance but had a much greater sensitivity (for more information on the history of the bolometer and its uses, see review by Richards 1994).

The first IR camera was invented by the Hungarian Physicist Kalman Tihanyi as a television camera that was used in Britain to detect an enemy aircraft in 1929 (Wimmer 2011). By 1960 the imaging of far infrared was developed using mercury-tellurium-cadmium detectors and was promoted exclusively for military use. It was not until 1956 that the first conventional IR camera was available for general use. After this, various improvements in digital circuitry and detection systems allowed companies to produce functional IR cameras at a reasonable cost making it available for various nonmilitary applications. For more details on the historical aspect of IR development, see reviews by Rogalski (2010) and Corsi (2010). For an extensive treatise on the principles underlying IR detection, see Gaussorgues (1994).

As resolution of thermal imaging improved and cost became more affordable, many uses for the technology became popular. This was primarily due to IR being a noncontact measurement process which makes it noninvasive to biological tissues, unlike the use of sensors that had to be applied to or inserted into the subject. One of the most significant uses was in the medical/veterinary field where early diagnosis of many illnesses could be made. For a review of medical applications for IR thermography, see a review by Lahiri et al. (2012). Other uses include building inspection to visualize areas devoid or deficient in insulation or maintenance issues such as poor electrical connections where the generation of heat occurs. For a review of these and other uses including the military usage in security and target acquisition, see review by Usamentiaga et al. (2014).

7.2 Freezing in Plants

The ability of plants to survive freezing is dependent on a number of interacting factors which make freezing tolerance one of the most complex problems facing plant physiologists, breeders, and growers. Indeed, a plethora of genes and/or DNA markers have been associated with the ability of plants to cold and/or freeze-acclimate, which can provide vital protection from ice nucleation (Hayes et al. 1993; Cai et al. 1994; Thomashow 1999; Brouwer et al. 2000; Reinheimer et al. 2004; Herman et al. 2006). Ice formation in plants can have devastating effects, particularly if a freezing event is preceded by supercooling that produces a critical displacement of temperature from the equilibrium (Olien 1967). The energy released when freezing is initiated under such conditions can be fatal to many plant tissues. However, tissues differ in their effect on survival of the whole plant. For example, leaves and roots in winter cereals such as rye, wheat, barley, and oat can be killed by freezing, but if meristematic tissues survive, the plant will regrow and produce a crop the following spring (Olien and Marchetti 1976; Tannino and McKersie 1985; Livingston et al. 2005). On the other hand, reproductive tissues of many species such as fruit trees are particularly susceptible to freezing injury (Wisniewski et al. 2008). If these plant parts are injured by freezing, the plant may survive, but complete loss of yield can result.

Important considerations in characterizing freezing resistance are nucleating agents, sites of nucleation (Wisniewski et al. 1997; Hacker and Neuner 2007), anatomical and chemical barriers to ice propagation (Aloni and Griffith, 1991; Zamecnik et al. 1994), speed of ice propagation, age, and/or size-dependency in order of freezing (Kaku and Salt 1968; Paerce and Fuller 2001). An accurate understanding of these processes is dependent on tissue-specific analysis that relies on considerable ingenuity as well as state-of-the-art technology to provide the degree of precision necessary to understand these processes accurately.

Various analytical methods have been used to investigate freezing patterns in a variety of plant species. Thermocouples attached to and/or embedded within plants is a common method that has been used for many years (discussed by Pearce and Fuller 2001). While thermocouples enable the detection of an increase in temperature when ice forms, it does not permit identification

of the precise location of ice initiation. In addition, thermocouples inserted into plant tissues can be a source of extrinsic nucleation (Fuller and Wisniewski 1998). An array of thermocouples (differential thermal analysis) can narrow the region of initiation but still cannot confirm the exact initiation point (Stier et al. 2003). Isothermal calorimetry can accurately determine a precise amount of heat given off during freezing but cannot provide any information as to which tissues had frozen nor where freezing was initiated (Livingston 2007). Freeze-fracture electron microscopy (e.g., Ball et al. 2002) can provide information on damage caused by ice in frozen tissues. Nuclear magnetic resonance (NMR) can quantify the amount of liquid water in specific tissues (Gusta et al. 1979; Millard et al. 1995; Ishikawa et al. 2016) and identify which tissues remain unfrozen. However, logistical considerations and expense preclude its routine use. With NMR it would not be possible to image whole plants and trees, nor would it be possible to monitor freeze events under natural conditions. While IRT has limitations, primarily that it cannot image the interior of plants, its versatility has allowed researchers to understand freezing and thawing of specific tissues, at the scale of the whole plant, in ways that have not been possible until now. It could be said that IRT is "tissue-specific calorimetry" and it has arguably given researchers more insight into tissue-specific freezing processes than any other technique.

7.3 IRT in Plants

The use of infrared thermography (IRT) to study freezing in plants is based on the principle that for liquid water to become ice, it must give up internal energy, which it does in the form of heat. At constant pressure this heat is 333 J/g of water (CRC Handbook Chem and Physics 1995). Provided the water does not undergo supercooling, the heat given up by the water at 0 °C causes a phase change from liquid to solid without changing the temperature, hence the term "latent heat." This heat, which is essentially released to the environment, is detectable in the IR region of the electromagnetic spectrum and is the basis for IRT in the study of freezing in plants. Using IR thermography to determine exact point of ice initiation is only possible if the resolution of the image is great enough. Due to limits of resolution, earlier technology allowed the detection of only very general regions where ice formation was initiated.

The first published use of IRT to study freezing in plants was by Ceccardi et al. (1995). In that study they visualized ice formation on the surface of excised jojoba leaves (Fig. 7.1). As Fig. 7.5 shows the resolution of the camera was poor, as compared to newer cameras. Because of the poor resolution, they were unable to determine the precise point of nucleation or exact tissues within which ice formation progressed. However, they successfully imaged temperature differences of freezing events in wet vs. dry plants. In addition, they were able to identify the general location on leaves where freezing events were initiated and determined that portions of leaves supercooled to a greater extent than others (Ceccardi et al. 1995).

Two years later Wisniewski et al. (1997) published an IR imaging study at higher resolution that showed the initial site of ice inoculation in bean leaves, peach and apple flowers, and rhododendron stems (Fig. 7.2). They were able to detect freezing of droplets that were as small as 0.5 uL, and they reported temperature measurements using the camera that were as accurate as that obtained by thermocouples (Wisniewski et al. 1997). They were also able to confirm the ability of ice+ bacteria to initiate freezing, and they reported the potential presence of intrinsic nucleating agents within woody plants.

7.4 Freezing Patterns in Plants Discovered by IRT

7.4.1 Freeze Inoculation

Freeze inoculation is the process whereby freezing begins in the plant. This can occur in a homogenous manner, which is essentially spontaneous freezing, or it can occur heterogeneously

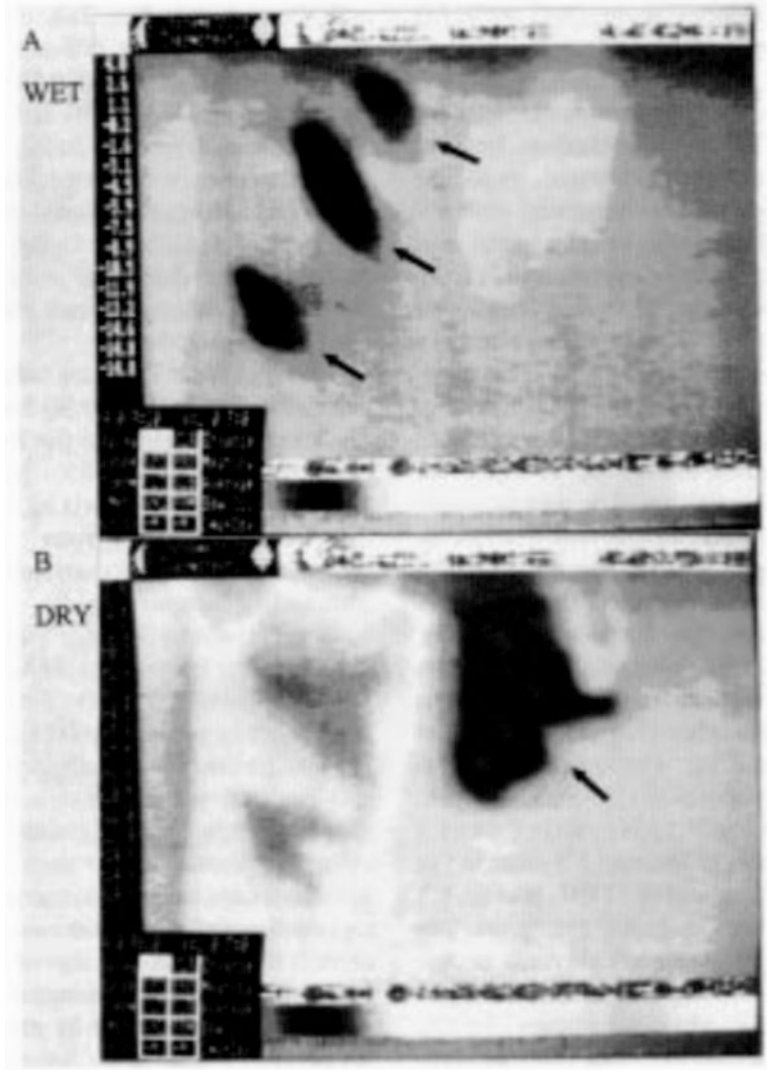

Fig. 7.1 Wet (**a**) and dry (**b**) jojoba leaves frozen to −12 °C under controlled conditions. Wet tissues froze at −8 °C and dry at −10 °C. In this case darker areas were warmer indicating that the black regions were areas of the leaf that had frozen. The camera used was a Model 600 IR Imaging Radiometer by Inframetrics. Note the poor resolution of images as compared to those generated by more recent cameras as shown in Fig. 7.6). This is the first published infrared image showing freezing in plants (Ceccardi et al. 1995)

as a result of an ice nucleation active substance either within or outside the plant. The homogenous nucleation temperature for water is −38 °C, and since most plants freeze at a considerably warmer temperature than this, it is difficult to demonstrate anything but heterogeneous nucleation in plants (Wisniewski et al. 2014). The exact tissue in which nucleation occurs is important because gene expression at the site of freeze initiation can give unprecedented insight into inoculating agents and/or anti-freezing agents. Identifying the genetic components of freezing resistance mechanisms can provide a basis for improving the ability of plants to survive freezing using marker-assisted selection (Hayes et al. 1993; Wooten et al. 2008), genetic transformation (Honjoh et al. 2001; Shou et al. 2004), or convention plant breeding (Livingston III et al. 2004).

Wisniewski et al. (1997) inoculated bean leaves with the ice+ *Pseudomonas syringae* bacteria and observed droplets containing the strain to freeze before droplets of plain water. They were not only able to follow the progression of

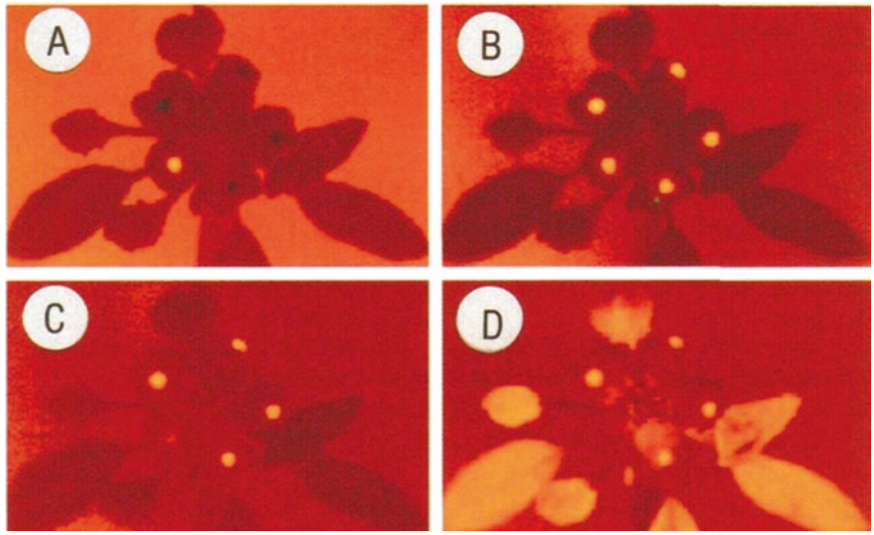

Fig. 7.2 IR monitoring of freezing in a detached apple flower. In this case the lighter color indicated a higher temperature (opposite of that in Fig. 7.1). Single yellow dot in (**a**) is a frozen droplet of *Pseudomonas syringe* culture to induce freezing. In (**b**), all droplets containing bacteria had frozen, but the plant did not freeze until (**d**) when the temperature was presumably lower. Note the much higher resolution image than in Fig. 7.1. The camera used in this analysis was a Model 760 Imaging Radiometer by Inframetrics. While the resolution in this image is considerably better than in Fig. 7.1, it is still poor as compared to more recent images as in Fig. 7.6 (Wisniewski et al. 1997)

freezing throughout the leaf but determined that once freezing was initiated in the inoculated leaf, ice propagated into the stem and throughout the entire plant (Fig. 7.3). They performed similar experiments on peach stems and flowers and determined that ice formation began in cortical tissue and then moved into xylem and pith (Wisniewski et al. 1997).

Fuller and Wisniewski (1998) subsequently monitored freezing in potato and cauliflower and found that the rate of ice propagation throughout the plant depended on the degree of supercooling. If the shoot initiated freezing at −2 °C, the entire stem took over 2 min to freeze. However, if the shoot supercooled to −6 and then froze, it took only about 10 s for the entire stem to freeze. The software of the system allowed them to determine that the temperature of the stem increased by approximately 2 °C. Importantly, they determined that the primary site of ice initiation was at the point of attachment of thermocouples. Thermocouples have been used for many years to monitor freezing in plants, and until IRT it was not known that this would occur.

Stier et al. (2003) reported that IRT allowed them to determine that in perennial ryegrass, roots always froze first followed by crowns and lower shoots and leaves froze last. In addition to the sites of freeze initiation, they determined that ice propagated at a rate of several centimeters per second in roots while freezing occurred more slowly in crowns.

Gusta et al. (2004) found that acclimated canola leaves with a lower water content initiated freezing more slowly with a "gradual progression to the main midvein." They found that cold-acclimated plants froze much more slowly and in a two-step process than non-acclimated plants. They state that the use of IRT allowed them to visualize the impact of solutes and water content on freezing in Canola (Gusta et al. 2004).

Using IRT in wheat plants in the reproductive phase, Livingston et al. (2016) found that soil and roots always froze first and freezing progressed from the bottom of the plant to the top, even though leaves cooled much more quickly and were always several degrees cooler than the soil. This was confirmed under natural conditions in

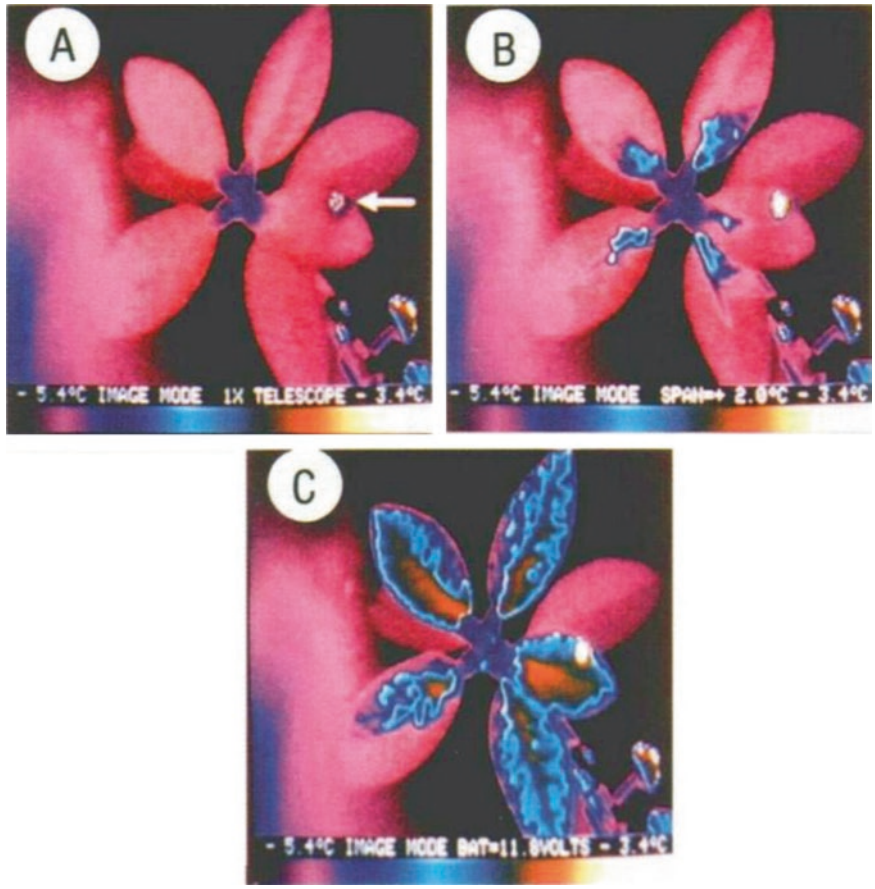

Fig. 7.3 Freezing of detached terminal tissue of rhododendron. Arrow in (**a**) indicates a droplet of *P. syringe* which has frozen in (**b**). Despite the droplet freezing, freezing of the plant began in the stem and spread upward into the leaves. Lighter blue color in this image indicated a higher temperature, showing where ice initiation began and spread through the leaves (Wisniewski et al. 1997)

both vegetative plants in the fall and in plants in a reproductive growth stage in the spring (unpublished observations).

For a concise review of ice inoculation, see Wisniewski et al. (2014), and for an exhaustive review of the subject, see the volume edited by Lee et al. (1995).

7.4.2 Barriers to Freezing

Barriers to freezing prevent ice growth into specific tissues in plants allowing the unfrozen tissues to supercool. These barriers are most commonly categorized as anatomical, but they can also be thermal or chemical (Wisniewski et al. 2014). It would be difficult to definitely categorize a particular barrier as strictly one kind or the other, and in many cases it is likely that more than one factor is involved in restricting the spread of ice. Ishikawa et al. (2016), using MRI, describe significant supercooling of anthers and ovules of flowering dogwood down to −14 to −21 °C. They suggest either the absence of ice inoculating activity, chemical substance that may stabilize supercooling, anatomical barriers, or a vapor phase barrier. Apart from this example, other freeze barriers were identified using IRT.

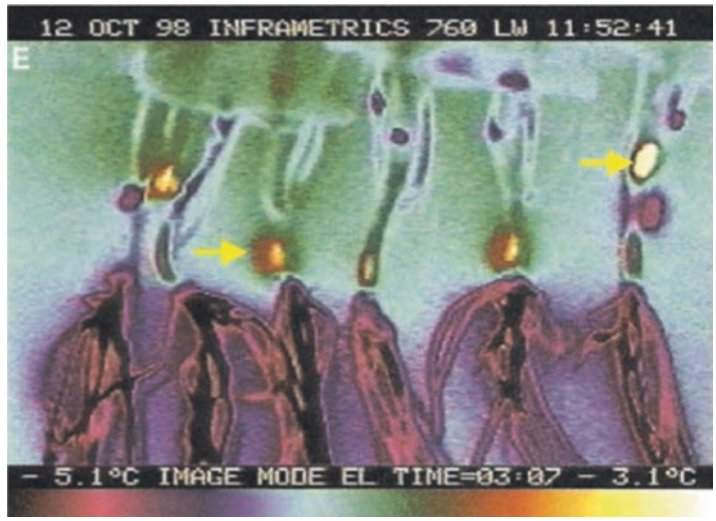

Fig. 7.4 Freezing in perennial ryegrass showing roots having been frozen but the upper part of the plant was just beginning to freeze. This demonstrated a barrier to freezing at the base of the crown. Once ice was initiated in the crown, a second barrier delayed freezing into leaves. Arrows indicate regions where droplets containing *P. syringe* had frozen. The *P. syringe* droplets froze about 3° lower than the roots, suggesting an intrinsic nucleating agent associated with root tissue (Stier et al. 2003)

Stier et al. (2003) attributed two regions of perennial ryegrass that appeared to freeze more slowly to barriers in an IRT analysis. They reported that roots always froze first but that freezing was inhibited once ice formation moved into the crown (Fig. 7.4). Once in the crown freezing progressed much more slowly and appeared to act as a barrier to ice propagating into leaves. Once ice began to form at the base of leaves, it moved quickly to the collar (junction between the leaf sheath and leaf proper) and occasionally appeared to slow at that point. While they did not conduct an analysis to determine the nature of the putative barriers, they cite Zamecnik et al. (1994) who proposed that the disperse nature of xylem as it transitions from roots into shoots should impede the growth of ice. In addition Aloni and Griffith (1991) demonstrate anatomical features in the root-shoot junction of selected cereal crops that could function as barriers to ice growth.

Pearce and Fuller (2001) describe similar findings of probable barriers in the crown tissue of barley and also cite Zamecnik et al. (1994) and Aloni and Griffith (1991). They found that the putative barrier in the crown of barley was more effective at a mild freezing temperature and that at lower temperatures, the delay of the spread of ice into leaves was almost ineffective. They suggested that the role of the barriers in cereals was to prevent freezing within the crown rather than to prevent leaves from freezing (Pearce and Fuller 2001).

Hacker et al. (2011) describe supercooling in the flowers of alpine cushion plants but demonstrated fairly conclusively that the barrier promoting supercooling in the flowers was thermal in nature and not structural. When putative thermal barriers were removed, "ice propagated unhindered throughout the whole cushion" (Hacker et al. 2011). On the other hand, Kuprian et al. (2016) provided convincing evidence for an anatomical barrier in the pedicel of Calluna (Fig. 7.5). Once the barrier was demonstrated using IRT, histological analysis found that pit apertures in cells within the zone of ice restriction

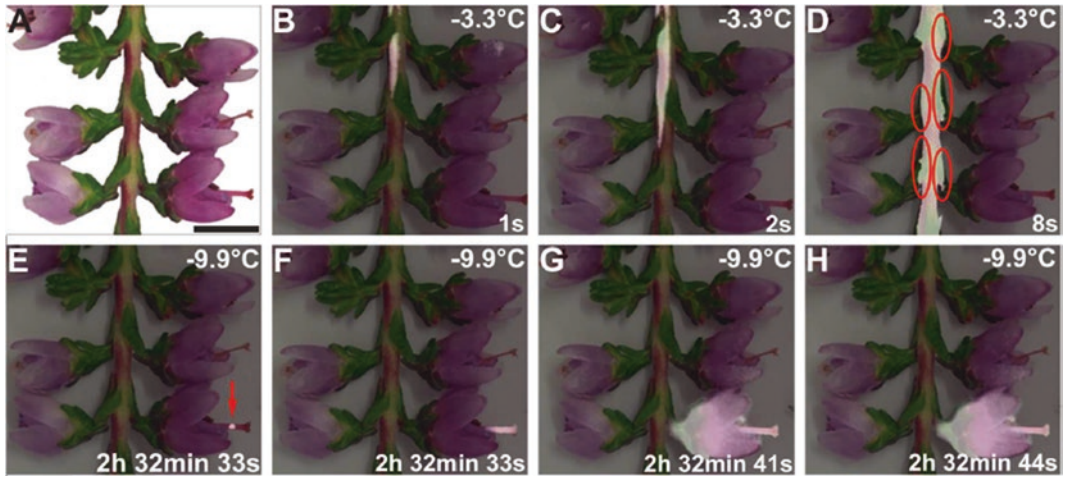

Fig. 7.5 Progression of freezing in a reproductive shoot of *Calluna vulgaris*. (**a**) Is the visible image of the tissue. A reduced opacity of the visible image was overlaid with the IR image to improve visibility. Lighter color is a warmer temperature, indicating tissues freezing. (**b**–**h**) Shows progression of freezing along with the temperature (upper right corner) and the time after inoculation of the stem by *P. syringe* (lower right corner). Note the considerable delay between D when the vegetative portion of the stem had frozen and E (−6.3 °C lower) when freezing began on the surface of the style (red arrow). All other flowers froze one at a time at different temperatures (not shown); the last flower to freeze did so at −15 °C. These observations led to a histological analysis of the stem-pedicel junction and the identification of barrier-like anatomical structures. Note the considerable improvement of resolution in these images as compared to Figs 7.1, 7.2, 7.3, and 7.4. Camera used was a Model T650sc by FLIR Systems (Kuprian et al. 2016)

were significantly smaller than in the vegetative stem where ice was able to propagate without obvious restriction. They document other anatomical features of the putative barrier such as a reduced number of "conducting units" in xylem as compared to vegetative tissue (Kuprian et al. 2016).

Livingston et al. (2016) found several regions in the stems of wheat in the reproductive stage of growth where freezing was restricted, one at the base of the plant at the root-shoot junction which corroborates the findings of Stier et al. (2003) and Pearce and Fuller (2001). In addition, they observed a restriction in ice propagation at a node just beneath the flowering head of the plant (Fig. 7.6). This point of restriction in freezing was described by Single and Marcellos (1981) as a "nodal block," and they stated that it was able to prevent freezing down to −7 °C. Fuller et al. (2007) described a similar barrier in wheat stems. Livingston et al. (2016) determined that when crown and node barriers were breached by ice, the plant always died and heads were not able to mature. However, if plants supercooled, the vegetative tissues survived, but the florets had varying degrees of sterility. In fact, they found significant differences in wheat genotypes for seed set in plants that had supercooled. Sterility of florets in supercooled wheat was reported by Fuller et al. (2009), but differences between genotypes at the same growth stage were not reported.

In addition to freezing, thawing patterns identified using IRT were reported (Livingston et al. 2016). In that study they determined that while plants froze in a tissue-specific manner over a range of temperatures, the same plants thawed strictly from the top of the plant to the bottom,

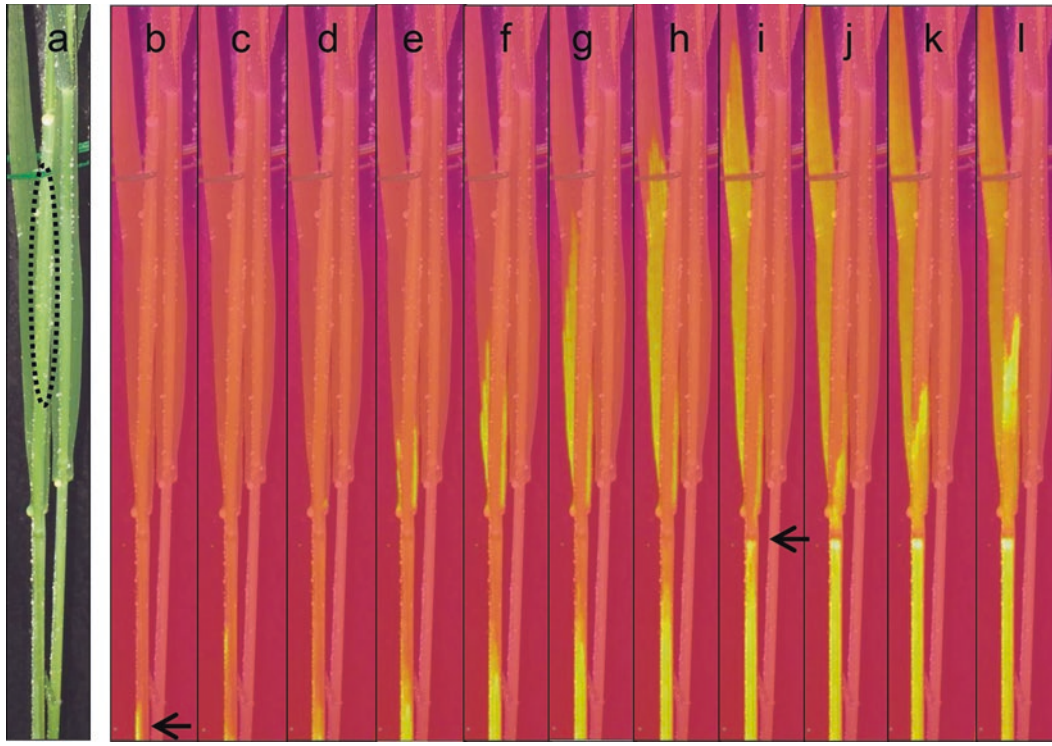

Fig. 7.6 A sequence showing the progression of freezing in the reproductive stem of wheat; two plants are shown. (**a**) is the visible image with the immature head still within the boot, shown in the dotted, elongated oval. The visible image with the opacity reduced was overlaid with the IR images to improve visibility in (**b**–**l**). Freezing was initiated spontaneously (no external inoculum) at the base of the stem. Arrow in b indicates where ice formation had entered the image; yellow color is warmer, indicating freezing. "b" through "l" are images taken 1 s apart. Note the delay in freezing the node (arrow) in "i" suggesting a barrier at this point. The plant to the right remained supercooled throughout the test. Camera used was a Model T620 by FLIR Systems. Resolution in this image is considerably better than that from earlier cameras as shown in Figs. 7.1, 7.2, 7.3, and 7.4. Note how the improvement in resolution allows observation almost to the cellular level (Livingston et al. 2016)

and the entire plant thawed at nearly the same temperature (Livingston et al. 2016). This indicated that prior to freezing, water was sequestered in various tissues with barriers (anatomical, chemical, temperature) of varying capacity to resist freezing, but once frozen, no such compartmentation was present to prevent ice from melting. With the plant surrounded by a background temperature just above freezing, the tissues that had frozen had not reabsorbed the latent heat needed to melt, so the frozen tissues remained at 0 °C and were darker than the background until they melted (Fig. 7.7).

7.5 Conclusion

While this is not an exhaustive review of all IRT studies in plants, it is hoped that the examples given here confirm the invaluable contribution IRT has provided to freezing research in plants. As IR technology, as well as other nondestructive techniques advance, it gives one hope that the exceptionally complex nature of freezing tolerance in plants will be understood, at least to the point where intelligent decisions can be made regarding conventional breeding schemes and/or genetic transformation.

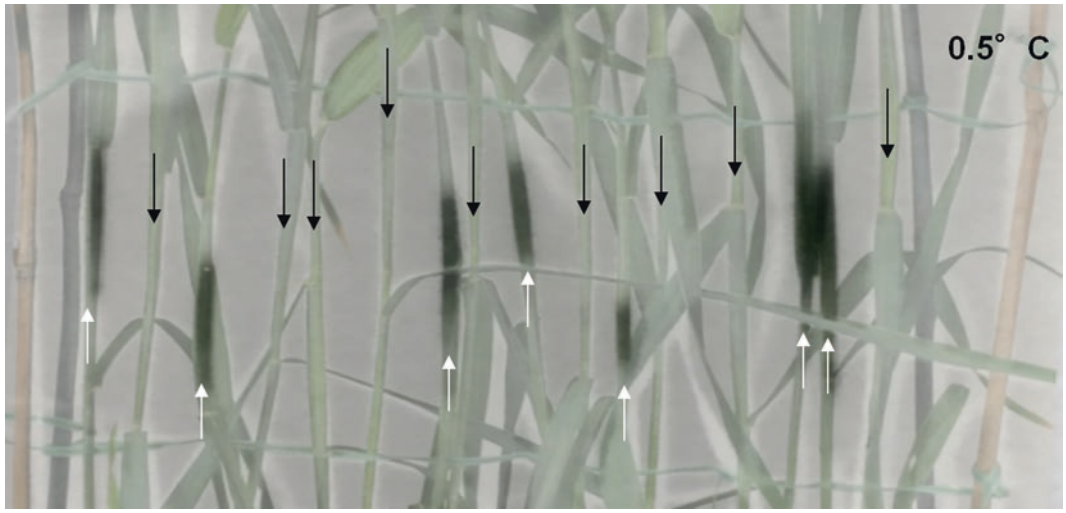

Fig. 7.7 A completed freeze test of 2 wheat cultivars with 8 plants per cultivar for a total of 16 plants. These plants were frozen to −6 °C and then allowed to thaw at 0.5 °C. The darkened regions of the stem are heads within the boot that had frozen and still have ice present even though the rest of the plant had equilibrated with the background and is therefore similar in color. White arrows pointing upward indicate heads that had frozen and still had ice in them at 0.5 °C. Black arrows pointing downward are heads of plants that never froze and therefore contained liquid water at the same temperature as the 0.5 °C background. The observation of the supercooled heads led to the discovery that wheat plants that supercool will become sterile even though they do not freeze, and while plants may appear undamaged, yield can be significantly reduced. Differences between genotypes in susceptibility to freeze damage were identified in this study (Livingston et al. 2016)

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Mechanism of Overwintering in Trees

8

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Abstract

Boreal trees possess very high freezing resistance, which is induced by short-day length and low temperatures, in order to survive severe subzero temperatures in winter. During autumn, cooperation of photoreceptors and circadian clock system perceiving photoperiod shortening results in growth cessation, dormancy development, and first induction of freezing resistance. The freezing resistance is further enhanced by subsequent low temperature during seasonal cold acclimation with concomitant changes in various morphological and physiological features including accumulation of sugars and late embryogenesis abundant proteins. The mechanism of adaptation to freezing temperatures differs depending on the type of tissue in boreal trees. For example, bark, cambium, and leaf cells tolerate freezing-induced dehydration by extracellular freezing, whereas xylem parenchyma cells avoid intracellular freezing by deep supercooling. In addition, dormant buds in some trees respond by extraorgan freezing. Boreal trees have evolved overwintering mechanisms such as dormancy and high freezing resistance in order to survive freezing temperatures in winter.

Keywords

Tree · Cold acclimation · Dormancy · Day length · Temperature sensing · Extracellular freezing · Deep supercooling · Extraorgan freezing

Abbreviations

ABA	Abscisic acid
CCA1	Circadian clock associated 1
CO	Constans
CRY	Cryptochrome
DREB1/CBF	Dehydration-responsive element-binding 1/C-repeat binding factor
DTA	Differential thermal analysis
EC	Evening complex
ELF	Early flowering
ER	Endoplasmic reticulum
FT	Flowering locus T

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FTL	Flowering locus T/terminal flower 1-like
LD	Long day
LHY	Late elongated hypocotyl
LT	Low temperature
LTE	Low-temperature exotherm
LUX	Lux arrhythmo
MPL	Multiplex lamellae
PHY	Phytochrome
PRR	Pseudo-response regulator
SD	Short day
TOC1	Timing of CAB2 expression 1
WT	Wild type
XRPC	Xylem ray parenchyma cells

8.1 Introduction

Trees are perennial vascular woody plants with lignified secondary cell walls; they have certain specific morphological and physiological features that differ from those of annual herbaceous plants (Pallardy 2008). Living a long life, trees enlarge the size of their wood body because of secondary growth that produces secondary xylem and phloem via the cambial layer activity in the trunk, branches, and roots. The longevity and huge body of trees are directly associated with the accumulation of biomass. Because trees perennate in their habitats, their adaptability to environmental changes is necessary (Larcher 2003). Besides phenology and the growth process of trees, which are synchronized with climatic rhythms, flexible responses to environmental stimuli are essential for trees owing to their longevity in their habitats. The ability to overwinter is a physiological feature of trees, and it is responsible for the fluctuation of day length and ambient temperatures (Sakai and Larcher 1987). The overwintering ability involves the dormancy development and cold acclimation (Welling and Palva 2006; Rohde and Bhalerao 2007; Preston and Sandve 2013). From autumn to winter, tree cells under various morphological and physiological changes and these cellular changes during seasonal cold acclimation lead to the development of freezing resistance in trees. In particular, boreal trees possess high freezing resistance to cope with severe freezing temperatures during the winter, and this ability enables them to live for many decades through severe winters even in high-latitude regions. Freezing resistance may be an important factor that affects the distributing of trees in cold climate regions.

Freezing resistance is primarily induced by short-day (SD) length along with growth cessation and dormancy development, and it is further induced by subsequent low-temperature (LT) exposure during seasonal cold acclimation with dormancy release in buds (Welling and Palva 2006). Thus, in trees, overwintering is a complex and dynamic event because both dormancy and freezing resistance are involved. In this section, we introduce the physiological events of overwintering and their mechanisms in trees.

8.2 Photoperiodic Mechanism of Growth Cessation, Bud Set, and Freezing Tolerance

Perennial plants such as trees predict the upcoming season by perceiving fluctuations in photoperiod and temperature throughout the year (reviewed in Tanino et al. 2010; Cooke et al. 2012; Singh et al. 2017). Trees growing in temperate and boreal zones must prepare to survive winter because extreme freezing temperature in winter is one of the most fatal conditions of the year (Weiser 1970; Sakai and Larcher 1987). In the seasonal transition from active to dormant, the initial phenological event is growth cessation and apical bud set in response to shortening day length (Weiser 1970). Following growth cessation, prolonged exposure to SD length induces increment in freezing tolerance. Thus, the annual photoperiodic change is a key environmental cue for trees to gauge the season and correct time during the day. Photoreceptors and the circadian clock are endogenous regulatory systems that perceive light conditions and measure photoperiods (reviewed in Singh et al. 2017). In this section, we discuss photoperiodic regulation of

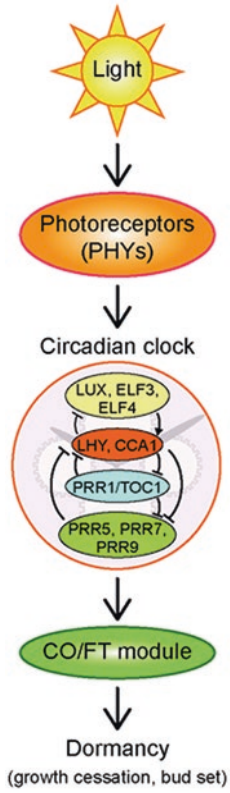

Fig. 8.1 Schematic diagram of the induction of seasonal dormancy in temperate and boreal trees. *CCA1,* CIRCADIAN CLOCK ASSOCIATED 1*; CO,* CONSTANS*; ELF,* EARLY FLOWERING*; FT,* FLOWERING LOCUS T*; LHY,* LATE ELONGATED HYPOCOTYL*; LUX,* LUX ARRHYTHMO*; PHY,* PHYTOCHROME*; PRR,* PSEUDO-RESPONSE REGULATOR*; TOC1,* TIMING OF CAB2 EXPRESSION 1.

growth cessation, bud set, and freezing tolerance in temperate and boreal trees (Fig. 8.1).

8.2.1 SD Induction of Overwintering Phenology

Trees growing in temperate and boreal regions follow an annual growth cycle: active growth from spring to summer, dormancy from autumn to winter, and growth reactivation in the following spring (Weiser 1970). Because meristematic and perennating tissues are exposed to extreme low temperatures during winter, trees have developed mechanisms to protect their tissues from frost damage (Weiser 1970; Sakai and Larcher 1987). In boreal trees such as birch (*Betula*), poplar (*Populus*), willow (*Salix*), and spruce (*Picea*), the preliminary environmental cue to initiate winter dormancy is the shortening of photoperiod (Pauley and Perry 1954; Heide 1974; Junttila 1980). When day length duration decreases below a critical value, trees initiate growth cessation and form apical buds, even under favorable conditions (Weiser 1970). The critical day length varies widely among inter- and intraspecies; northern genotypes show a longer critical day length compared to southern genotypes because of larger seasonal and daily differences in natural day length in northern latitudes (Pauley and Perry 1954; Heide 1974; Junttila 1980). Although photoperiodic fluctuation is a preliminary signal for the induction of seasonal dormancy, ambient temperature also affects the timing of growth cessation and bud set (Kalcsits et al. 2009; reviewed in Tanino et al. 2010). Concurrent with and subsequent to growth cessation, freezing tolerance begins to increase in overwintering tissues (Weiser 1970; Sakai and Larcher 1987). Hardwood trees such as *Betula* and *Populus* spp. acquire provisional freezing tolerance under SD conditions (Welling et al. 2002; Li et al. 2003a, 2005; Kalcsits et al. 2009), and their initial tolerance level is rapidly increased by subsequent exposure to low nonfreezing and freezing temperatures, resulting in maximum frost hardiness (Weiser 1970; Sakai and Larcher 1987).

8.2.2 Photoreceptor Involvement in Overwintering Phenology

Plants have a gamut of photoreceptors that enable adaptation to changing light environment. Blue and UV-A lights are perceived by several receptors such as cryptochromes (CRYs), phototropins (PHOTs), and ZEITLUPE (ZTL) family members [ZTL, FLAVIN-BINDING KELCH REPEAT F-BOX 1 (FKF1), and LOV KELCH PROTEIN2 (LKP2)] (reviewed in Christie 2007; Chaves et al. 2011; Ito et al. 2012). In the past decade, thale cress (*Arabidopsis thaliana*) molecular genetics identified a novel UV-B

photoreceptor, UV RESISTANCE LOCUS 8 (UVR8) (Rizzini et al. 2011). Although these photoreceptors seem to be conserved in a broad range of land plants (Cloix et al. 2012; Nystedt et al. 2013; Li et al. 2014; Li and Mathews 2016), a detailed study of their association with overwintering phenology has not been performed in boreal and temperate trees.

Knowledge regarding the involvement of photoreceptors in seasonal growth patterns has progressed in red and far-red light photoreceptors, phytochromes (PHYs), in the model tree *Populus* (Fig. 8.1). Transgenic poplars that strongly express the oat *phyA* gene are insensitive to SDs even under 6 h of day length, whereas wild-type (WT) plants show growth cessation and terminal bud set under a 14-h photoperiod (Olsen et al. 1997). Moreover, freezing tolerance, induced by prolonged SD exposure, does not increase in *phyA*-overexpressing poplars (Olsen et al. 1997; Welling et al. 2002). Kozarewa et al. (2010) report that *phyA* knockdown poplars undergo growth cessation and form terminal buds earlier than WT plants at critical day length of 15.5 h, indicating that seasonal dormancy and freezing tolerance induced by SDs are mediated by the phyA light-signaling pathway. In addition, quantitative trait loci analyses demonstrate that *phyB*s in *Populus* are candidate genes for the involvement of photoperiodic perception and are associated with the timing of terminal bud formation (Frewen et al. 2000; Ingvarsson et al. 2006). In softwood trees, *phyN* and *phyO* (orthologous to angiosperm *phyA* and *phyC*) and *phyP* (orthologous to angiosperm *phyB* and *phyE*) are conserved (Li et al. 2015); however, association of coniferous PHYs with the induction of seasonal dormancy remains an important open question.

8.2.3 Circadian Clock Involvement in Overwintering Phenology

Plants have an endogenous time-keeping mechanism, the circadian clock, to gauge daily and seasonal environmental changes. In the past few decades, molecular genetics and mathematical analyses in *A. thaliana* have uncovered a molecular network of the plant circadian clock (Pokhilko et al. 2012; Adams et al. 2015). The core components of the circadian clock are two *MYB* genes, *LATE ELONGATED HYPOCOTYL* (*LHY*) and *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*), and a *PSEUDO-RESPONSE REGULATOR 1/TIMING OF CAB2 EXPRESSION 1* (*PRR1/TOC1*). These genes form the feedback loop; the morning-acting *LHY* and *CCA1* directly repress the expression of evening-acting *PRR1/TOC1*, and *PRR1/TOC1* in turn suppresses the expression of *LHY* and *CCA1* (Wang and Tobin 1998; Alabadí et al. 2001; Kim et al. 2003) (Fig. 8.1). *LHY* and *CCA1* are involved in another feedback regulation with *PRR5*, *PRR7*, and *PRR9*, homologs of *PRR1/TOC1*, which show diurnal and sequential expression patterns from dawn in the following order: *PRR9* → *PRR7* → *PRR5* → *PRR1/TOC1* (Makino et al. 2001; Nakamichi et al. 2005). *PRR5*, *PRR7*, and *PRR9* are repressors of *LHY* and *CCA1*, and their expression is negatively regulated by *LHY*, *CCA1*, and *PRR1/TOC1* (Huang et al. 2012; Adams et al. 2015). Furthermore, gene expression of *LHY* and *CCA1* is promoted by evening complex (EC), which is built up by the MYB-like transcription factor LUX ARRHYTHMO (LUX), EARLY FLOWERING 3 (ELF3), and ELF4 (Nusinow et al. 2011; Nagel and Kay 2012; Adams et al. 2015). EC components, in turn, are repressed by LHY and CCA1. The mathematical model of the plant clock is organized by complicated interactions among core components and is constantly updated because other circadian clock-related genes such as *GIGANTEA* (*GI*), *REVEILLE* (*RVE*), *NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED* (*LNK*), and *CCA1 HIKING EXPEDITION* (*CHE*) are directly and/or indirectly involved in the circadian clock network (Pokhilko et al. 2012; Adams et al. 2015).

In trees, molecular network of the circadian clock is advanced in *Populus*. The *Populus* genome retains an ortholog of clock-related genes such as *LHY/CCA1*, *PRRs*, *LUX*, *ELF3*, and *ELF4* (Takata et al. 2009, 2010; Johansson et al. 2015). Gene expression analyses using transcriptomics and qPCR demonstrated typical diurnal expression patterns of many clock genes in

Populus, although *PRR1/TOC1* showed an earlier phase expression than the *Arabidopsis* ortholog (Takata et al. 2009, Ibáñez et al. 2010). Likewise, orthologs of core clock genes (*LHY*, *PRR1/TOC1*, *PRR5*, *PRR7*, and *PRR9*) are conserved in the chestnut tree (*Castanea sativa*) and show a typical diurnal expression pattern in a serial manner: *CsLHY* → *CsPRR9* → *CsPRR7* → *CsPRR5* → *CsPRR1/TOC1* (Ramos et al. 2005; Ibañez et al. 2008). Transgenic approaches in *Populus* have helped to elucidate that LHYs (LHY1 and LHY2) and PRR1/TOC1 negatively regulate the expression of *PRR5s* and *LHYs*, respectively (Ibáñez et al. 2010). These findings reveal a high degree of conservation of the plant clock network between hardwood species and in *A. thaliana*.

Studies of the clock system in softwood species have progressed in Norway spruce (*Picea abies*) (Karlgren et al. 2013b; Gyllenstrand et al. 2014). Despite having fewer clock genes than *A. thaliana*, *Picea* orthologs show a typical diurnal expression under long-day conditions, which is similar to that in angiosperms: *PaCCA1* → *PaPRR7* → *PaPRR1/TOC1*. Rhythmic expression of the genes is, however, rapidly dampened under constant light and constant dark conditions, suggesting that the gene regulatory network of a clock system in conifers may be distinct from that in angiosperms.

In temperate and boreal trees, the circadian clock system links the fluctuation of natural photoperiod and seasonal growth cessation. Transgenic poplars, in which either LHYs or PRR1/TOC1 expression is reduced by RNA interference, demonstrate approximately 1 h shorter critical day length for growth cessation and earlier bud set as compared to the WT trees, suggesting that the clock system plays a key role in determining critical day length (Ibáñez et al. 2010). Interestingly, the opposite effect on freezing tolerance is observed in the knockdown poplars; trees with lower *LHY* levels are more sensitive to freezing temperatures, whereas those with lower *PRR1/TOC1* levels have a higher cold hardiness than the WT trees. This is because the expression of DEHYDRATION RESPONSIVE ELEMENT-BINDING FACTOR 1/*C-REPEAT BINDING FACTOR* (*DREB1/CBF*) genes, master switches in cold-responsive gene expression, is controlled by the *Populus* clock system. In *A. thaliana*, the reciprocal regulation between DREB1/CBFs and the circadian clock contributes to the increased cold tolerance (Nakamichi et al. 2009; Dong et al. 2011; Chow et al. 2014). Although the investigation in the knockdown poplars indicated that the clock system is involved in the increase of freezing tolerance under LTs, it remains unclear whether the initial freezing tolerance induced by SD conditions is gated by the clock system.

The milestone with regard to seasonal growth cessation was the discovery of the engagement of *CONSTANS/FLOWERING LOCUS T* (*CO/FT*) regulatory module with this phenology in boreal and temperate trees. This module was identified to be a core pathway involved in promoting photoperiodic flowering in the long-day plant *A. thaliana* (Suárez-López et al. 2001). In *A. thaliana*, CO shows a diurnal rhythm, with peak expression before dusk under long-day (LD) conditions. The CO protein is stabilized under light conditions through photoreceptors such as phyA and CRYs, which leads to activation of *FT*, a mobile floral activator (Valverde et al. 2004; Liu et al. 2008). By contrast, *CO* exhibits peak expression in the dark under SD conditions; thus, the CO protein is unstable and cannot induce *FT* expression. *Populus CO*, as in *A. thaliana*, is expressed diurnally with peak expression in the afternoon, and it is a positive regulator of *FT* expression (Böhlenius et al. 2006). When the day length shifts from LDs to SDs, *CO* expression peaks under dark conditions, and the CO protein may be unstable. Consequently, *FT* expression is not induced under SD conditions, leading to growth cessation and bud set (Böhlenius et al. 2006; Hsu et al. 2011) (Fig. 8.1). Transgenic studies give insights into this model because *FT* knockdown poplars have a higher sensitivity to SDs, and *FT* overexpressed poplars are less or unresponsive to critical day length (Böhlenius et al. 2006; Hsu et al. 2011). Furthermore, the CO/FT model is well supported by the investigation of different *Populus tremula* populations across the latitudinal cline, which have unique and different critical day lengths (Böhlenius et al.

2006). *CO* expression peaks later (i.e., the peak expression will be preponed in the dark after midsummer) in *Populus* genotypes derived from a northern latitude which have a longer critical day length, compared to those from a southern latitude. Unlike the CO/FT regulatory module in *Populus*, *FLOWERING LOCUS T/TERMINAL FLOWER1*-Like (*FTL*) genes are induced after transfer to SDs in Norway spruce (*P. abies*) and Scots pine (*Pinus sylvestris*) (Gyllenstrand et al. 2007; Karlgren et al. 2013a; Avia et al. 2014). In addition, transgenic spruce trees overexpressing *FTL* show a higher induction of bud set and growth cessation compared to control plants under continuous light condition, suggesting that *FTL* may positively regulate the transition to seasonal dormancy in softwood trees (Karlgren et al. 2013a). Gymnosperm *FTL*s share a common ancestor with angiosperm *FT* and *FTL* and are functionally close to a floral suppressor *FTL* of *A. thaliana*, an antagonist of *FT* (Karlgren et al. 2011). Taken together, these findings imply that *FT* and *TFL* play key roles as integrators of seasonal growth cessation both in angiosperms and gymnosperms.

8.3 Physiological Changes in Woody Perennials During Cold Acclimation

In order to survive severe winters without snow cover, overwintering woody perennials have evolved complex mechanisms to acquire high freezing resistance by cold acclimation processes and for dynamic control of their freezing resistance based on environmental cues. Although a great deal of effort has been invested in investigating these mechanisms, several aspects remain unexplained. In this section, we have summarized the effect of day length and temperature on freezing resistance of tree cells. The role of physiological changes during cold acclimation, including signal transduction, gene expression, protein composition, sugar accumulation, and cell ultrastructure associated with increase in freezing resistance, is also discussed.

8.3.1 Induction of Freezing Resistance by SD, LT, and Their Combination

Under natural environmental conditions, seasonal cold acclimation of woody perennials is primarily induced by SD during autumn, but it is further enhanced by LT during extreme winter (Howell and Weiser 1970a). The influence of SD, LT, and their combination on the development of freezing resistance in tree cells has been the focus of many studies (Howell and Weiser 1970a; Christersson 1978; Junttila and Kaurin 1990; Taulavuori et al. 2000; Li et al. 2002; Welling et al. 2002). Researchers have shown that either SD or LT can significantly increase freezing resistance of trees. Their independent roles were supported by the findings of Welling et al. (2002), wherein an SD-insensitive transgenic hybrid aspen line overexpressing an oat *phyA* gene could increase freezing resistance under LT condition. Although the level of increase in freezing resistance induced by either SD or LT varies with regard to several factors, including plant species, tissues, and experimental conditions, some reports suggest that very high freezing resistance exceeding −40 °C is induced by either SD (Junttila and Kaurin 1990; Taulavuori et al. 2000) or LT (Howell and Weiser 1970a) in tree tissues. In many cases, however, a combination of SD and LT is required for complete cold acclimation (Howell and Weiser 1970a; Christersson 1978; Taulavuori et al. 2000). The sequential order and duration of different environmental conditions can also be important for optimum freezing resistance development (Christersson 1978). Exceptionally, stem tissues of Scots pine (*P. sylvestris*) demonstrated higher freezing resistance under SD condition (7-h photoperiod) with a high temperature (15 °C) than under SD condition with LT (2 °C) (Zhang et al. 2003).

8.3.2 Temperature Sensing by Trees for Cold Acclimation

In blue-green algae, membrane fluidity has been shown to play a significant role in sensing LT

(Murata and Los 1997), and histidine kinase Hik33 has been identified as a cold sensor which perceives changes in membrane fluidity as the signal in *Synechocystis* (Suzuki et al. 2000). Membrane fluidity might play a similar role in a temperature-sensing mechanisms of higher plants. However, temperature sensors had not been identified in higher plants until quite recently. In 2016, two reports identifying temperature sensors in *Arabidopsis* were published (Jung et al. 2016; Legris et al. 2016). The identified sensors were red/far-red light receptor PHY family proteins, and their expected temperature-sensing mechanism was completely different from that of blue-green algae, which relies on membrane fluidity. The temperature-sensing mechanism is involved in the rate of thermal reversion of PHY molecules from the active form (Pfr) to the inactive form (Pr). The conversion from Pfr to Pr is primarily mediated by far-red and red lights. Thermal reversion is another mediator of reversion from Pfr to Pr. The rate of light-independent reversion is affected by temperature. Currently, it is unclear whether PHY proteins play a role in the temperature-sensing mechanism for cold acclimation in plants. Even though PHYs may act as temperature sensors during cold acclimation, willow (*Salix sachalinensis*) cells harvested in autumn have been found to undergo increase in their cold hardiness under dark conditions (Sakai 1966), suggesting that PHYs are not the only temperature sensors for cold acclimation. The presence of freezing and chilling temperature-sensing system could also be explained by the temperature-dependent response of trees during the deacclimation process. Deacclimation processes are regulated by temperature rather than light signals (Howell and Weiser 1970b).

8.3.3 Hormonal Control of Cold Acclimation in Trees

The role of plant hormones in stress signaling in herbaceous plants is well established. Cold acclimation processes of trees are also expected to undergo hormonal regulation. The best-studied hormone associated with cold acclimation in trees is abscisic acid (ABA). Under conditions that increase freezing resistance, an increase in endogenous ABA level was observed in birch species (Welling et al. 1997, 2002; Rinne et al. 1998; Li et al. 2002). ABA is believed to influence cold acclimation in addition to growth cessation and dormancy (Welling et al. 1997). Exogenous ABA increased freezing resistance in birch trees (*Betula pendula*) and grapevines (*Vitis vinifera*) even in the absence of LTs (Li et al. 2003b; Karimi and Ershadi 2015). Furthermore, the ABA biosynthesis inhibitor fluridone decreased ABA levels and inhibited sufficient cold acclimation in birch trees (*Betula pubescens*) (Welling et al. 1997). The involvement of ethylene, gibberellin, and jasmonate in cold acclimation signaling via hormonal cross talk has also been suggested in herbaceous plants (Wingler 2015; Hu et al. 2017). In white spruce (*Picea glauca*), changes in expression levels of genes putatively associated with such hormone signaling were observed (El Kayal et al. 2011).

8.3.4 Gene Expression and Protein Accumulation During Cold Acclimation

During cold acclimation processes, many physiological changes that may be associated with alterations in gene expression occur in plant cells (Thomashow 1999). Generally, stress-response genes can be categorized as genes involved in signal transduction and those that encode proteins which directly participate in the protection of cells under stress conditions (Welling and Palva 2006). The best-studied gene families in cold acclimation regulatory pathways in plants are DREB1/CBF genes encoding transcription factors, which are often considered to be "master switches" of activation of a regulon of genes involved in cold acclimation (Thomashow et al. 2001). DREB1/CBF proteins recognize and bind to dehydration-responsive element/C-repeat (DRE/CRT) *cis*-acting elements of cold-regulated genes and activate their expression. DREB1/CBF families are well conserved in plant species

including woody perennials. DREB1/CBF orthologs have been cloned from several woody perennials (Welling and Palva 2006), and their induction in controlled LT conditions (Benedict et al. 2006; Chen et al. 2014; Leyva-Pérez et al. 2015) and during autumn (Schrader et al. 2004) was suggested. Constitutive expression of *Arabidopsis CBF1* gene (*AtCBF1*) resulted in a significant increase in freezing resistance of leaves and stems of non-acclimated hybrid poplars (Benedict et al. 2006). Interestingly, a gene set in stems of the non-acclimated transgenic poplar (perennial tissue) was different than that of the cold-acclimated stems and leaves of the WT poplar, although the gene set in the leaves (annual tissue) of the non-acclimated transgenic poplar was similar to that in acclimated tissues in WT plants, suggesting specific roles of DREB1/CBFs in the different annual and perennial tissues of woody perennials (Benedict et al. 2006). Furthermore, the expression responses of two of four poplar *DREB1/CBF* paralogs to LT were different between leaves and stems (Benedict et al. 2006). Recently, it was also suggested that DREB1/CBFs affected the induction of dormancy and increase in freezing resistance (Wisniewski et al. 2011).

Many studies have investigated changes in gene expression and protein composition associated with cold acclimation (Welling and Palva 2006). More recently, large-scale analytical methods called as transcriptomics (Schrader et al. 2004; Bassett et al. 2006; Maestrini et al. 2009; El Kayal et al. 2011; Galindo González et al. 2012; Chen et al. 2014; Fernández et al. 2015; Leyva-Pérez et al. 2015) and proteomics (Renaut et al. 2008; Galindo González et al. 2012) have been employed for molecular analysis of cold acclimation processes in woody and non-woody perennials. In these exhaustive studies, the induction of transcripts or proteins associated with stresses and defense mechanisms (Bassett et al. 2006; Renaut et al. 2008; Maestrini et al. 2009; El Kayal et al. 2011; Chen et al. 2014; Fernández et al. 2015), carbohydrate metabolism (Renaut et al. 2008; El Kayal et al. 2011; Galindo González et al. 2012; Chen et al. 2014), and signal transduction such as DREB1/CBFs (Maestrini et al. 2009; Chen et al. 2014; Fernández et al. 2015; Leyva-Pérez et al. 2015) were commonly reported.

Dehydrin is one of the well-studied stress-related protein groups induced during cold acclimation. Dehydrins are group 2 members of the late embryogenesis abundant protein family, which are highly hydrophilic (Hanin et al. 2011). The precise function of dehydrins is unclear, but their involvement in the development of dehydration tolerance in cells has been indicated. Dehydrins are induced by stresses that cause cellular dehydration, including drought, freezing, high salinity, and osmotic stress (Hanin et al. 2011). Cells in woody perennials undergo two types of dehydration during winter. The first type is caused by decrease in water content of perennial tissues during SD-induced acclimation (Welling et al. 2002). The second type is freezing-induced dehydration because of the difference between vapor pressures of extracellular ice and intracellular water (Sakai and Larcher 1987; Kuroda et al. 2003). Dehydrins exhibited protective effects on proteins under dehydration, freezing, and high-temperature stresses (Wisniewski et al. 1999; Drira et al. 2013). They have also been proposed to function as antifreeze proteins (Wisniewski et al. 1999) and radical scavengers (Hara et al. 2004). The important role of dehydrins in trees under freezing stress would be supported by the fact that ectopic overexpression of a citrus dehydrin gene in tobacco improved freezing tolerance and inhibited lipid peroxidation (Hara et al. 2003).

8.3.5 Other Physiological Changes During Cold Acclimation

The accumulation of soluble sugars is a well-known response to freezing, drought, salt, and osmotic stresses in plants. Changes in tissue sugar content of trees have often been investigated (Sakai and Larcher 1987; Sauter et al. 1996; Kasuga et al. 2007). In certain species, a correlation was observed between the contents of sucrose and raffinose family of oligosaccharides

and increase in freezing resistance of cells during winter (Sauter et al. 1996; Kasuga et al. 2007). Some putative roles of sugars in plant response to freezing stress in woody perennial cells are discussed in the next section.

Cellular structural changes have also been reported as another kind of physiological change during cold acclimation. The disappearance of large vacuoles and their replacement by small vacuoles filled with osmiophilic materials were observed in cortical and xylem parenchyma cells of poplar species (Sagisaka et al. 1990; Sauter et al. 1996). An increase in the number of protein bodies and oleosomes was also observed in both these types of cells (Sagisaka et al. 1990; Sauter et al. 1996). In contrast, the number of large starch granules in amyloplasts observed during autumn decreased by early winter (Sagisaka et al. 1990; Sauter et al. 1996), which may be attributable to conversion of starch to soluble sugars. Fujikawa and Takabe (1996) demonstrated ultrastructural alteration of the endoplasmic reticulum (ER) during freezing process in cortical cells of mulberry (*Morus bombycis*) harvested during winter and cold acclimation period (see the next section). Several studies have also reported ultrastructural changes in cell wall during cold acclimation. Callose deposition has been reported in cell wall of suspension-cultured tree cells (Wallner et al. 1986). In the xylem parenchyma cells of peach (*Prunus persica*), apparent loosening or a partial dissolution of portions of the amorphous layer on pit membranes, close to vessels, was observed during artificial deacclimation (Wisniewski and Davis 1989). It has also been suggested that the black cap structure covering the pit membranes of xylem parenchyma cells in the peach becomes more pronounced during winter (Wisniewski and Davis 1995). However, Kasuga et al. (2013) suggested that seasonal changes in the cell wall properties of xylem parenchyma cells that resist freezing stress by deep supercooling were not associated with fluctuation in the supercooling capability of cells. The roles of ultrastructural changes in cell wall during cold acclimation are still under discussion.

8.4 Response of Tree Cells to Subzero Temperatures

Freezing of intracellular water (intracellular freezing) causes lethal damage to cells when the growth of large ice crystals at prolonged subzero temperatures promotes the destruction of intracellular structures and endomembranes. Therefore, most cells in overwintering plant tissues resist intracellular freezing by extracellular freezing. In trees, cortical parenchyma cells adapt to subzero temperatures by extracellular freezing; conversely, xylem ray parenchyma cells (XRPCs) adapt by deep supercooling (Fig. 8.2). Both mechanisms are different because of the differential behavior of water movement at subzero temperatures in each cell. Furthermore, dormant buds of certain trees exhibit extraorgan freezing, which is a systematic response of a whole bud, unlike cellular responses such as deep supercooling and extracellular freezing (Fig. 8.3). In this section, the three types of freezing behaviors are introduced.

8.4.1 Extracellular Freezing

Extracellular freezing is a typical freezing behavior widely observed in herbaceous plants and in the leaf and bark tissues of trees (Sakai and Larcher 1987; Fujikawa 2016). The freezing resistance of bark tissues of trees inhabiting cool climate regions is much higher than that of herbaceous plants. The freezing resistance of bark tissues of cold-acclimated trees, such as Japanese white birch (*Betula platyphylla*) and larch (*Larix kaempferi*) of Hokkaido in winter, is extremely high, and bark tissues frozen at a slow cooling rate, as well as at natural rates, can survive treatment with liquid nitrogen.

When ambient temperatures gradually or naturally decline to subzero temperatures, apoplastic water is first frozen, and extracellular ice growth progresses in accordance with the vapor pressure gradient that is formed between extracellular ice and temporally supercooled intracellular water. Thus, cells undergo dehydration

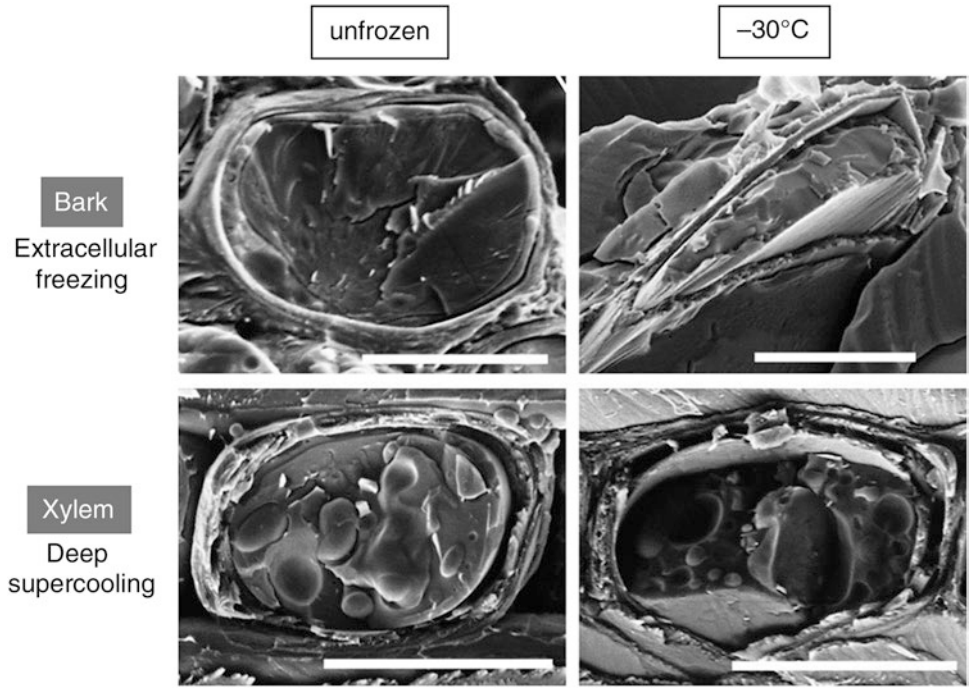

Fig. 8.2 Cryo-scanning electron microscopic analysis of freezing behavior of the cells in twig of seasonal cold-acclimated *Cercidiphyllum japonicum*. Scale bars: 10 μm

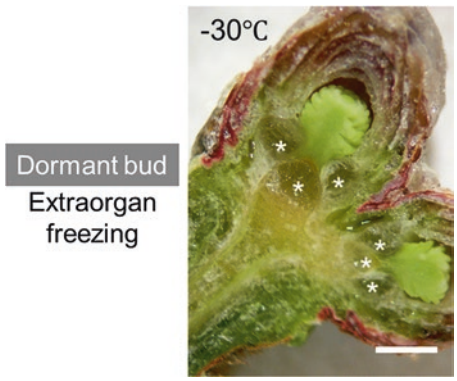

Fig. 8.3 Extraorgan freezing of dormant buds of *Abies sachalinensis*. At −30 °C, extracellular large ice crystals (*) were accumulated in the extracellular void space (ice sink) of bud and were not observed within primordia. Scale bar: 1 mm

because of extracellular ice growth until their water potential is equilibrated with that of the intracellular space across plasma membranes at ambient subzero temperatures. As the temperature declines, these cells shrink as a result of freezing-induced dehydration and undergo deformation because of extracellular ice growth occurring in the process of extracellular freezing (Fig. 8.2). Therefore, severe freezing-induced dehydration is caused by the excess freezing of plant cells, and it results in freezing injury of the cells, especially in the plasma membrane, which has been known as the primary site of injury (Steponkus 1984) because severe dehydration produces mechanical stress in cells (Gordon-Kamm and Steponkus 1984; Pearce and Willison 1985), and extracellular ice growth causes cellular deformation (Fujikawa and Miura 1986). Previous studies have demonstrated that irreversible changes in the ultrastructure of the plasma membrane are first caused in areas where the interaction of membrane lipids between the plasma membrane and intracellular membranes (or other sites of plasma membranes) is promoted by their extraordinary proximity, which is a result of significant shrinking and deformation by freezing-induced dehydration. These ultrastructural changes in plasma membranes, caused by

the interaction of membrane lipids between biomembranes brought about by excess freezing, were observed as the formation of hexagonal II phase transition of membrane lipids (Gordon-Kamm and Steponkus 1984) and/or aparticulate domains (Fujikawa and Steponkus 1990) followed by membrane fusion. Thus, it is believed that the membrane stability of plasma membranes during extracellular freezing is an important factor in resistance to freezing injury and the development of freezing tolerance in plant cells (Steponkus et al. 1993). Changes in lipid and protein compositions of plasma membranes during cold acclimation may be associated with increase in freezing tolerance (Uemura and Yoshida 1984; Steponkus et al. 1993; Zhou et al. 1994; Kawamura and Uemura 2003). Membrane lipids with highly hydrated polar head groups, such as phosphatidylcholine, may prevent close contact between endomembranes during extracellular freezing (Steponkus and Webb 1992). Some plasma membrane proteins induced by cold acclimation may also contribute to stabilization of plasma membranes as shown by transgenic studies using *Arabidopsis* (Uemura et al. 2006). Furthermore, other cellular factors such as accumulation of soluble sugars (Kasuga et al. 2007) and certain cold-inducible soluble proteins with potent cryoprotective effects may result in plasma membrane stability at subzero temperatures (Fujikawa et al. 2006). Several studies attempted to identify the contribution of cellular factors, such as CBF regulons, induced during cold acclimation in the development of freezing tolerance (see some reviews, Thomashow 1999; Welling and Palva 2006; etc.). For example, the overexpression of late-embryogenesis-abundant proteins, such as COLD-REGULATED 15a (COR15a), that accumulate in the chloroplast stroma of cold-acclimated *Arabidopsis thaliana* decreased during freezing injury of plasma membranes through the stabilization of chloroplast membranes (Artus et al. 1996). Soluble sugar accumulation in cells may contribute to depression in freezing point and reduction of dehydration and deformation. Also, highly concentrated soluble sugars may stabilize macromolecules, such as cell membranes, by substitution of water molecules on their surfaces (Crowe et al. 1988) or by interaction of vitrified sugars with membrane lipid (Koster et al. 1994) during severe freezing-induced dehydration.

In addition, it is thought that the endomembrane system is also associated with the stabilization of plasma membranes during extracellular freezing (Fujikawa and Takabe 1996). As mentioned in the previous section, morphological changes in ER were detected in bark tissues of mulberry during seasonal cold acclimation and extracellular freezing at subzero temperatures during winter. ER-derived vesicles accumulated during seasonal cold acclimation underwent fusion and formed multiplex lamellae (MPL) in the ER beneath plasma membranes because of freezing-induced dehydration at relatively high subzero temperatures in winter. MPL formation by fusion of ER-derived vesicles beneath the plasma membrane was also induced by treatment with hyperosmotic solution. Production of MPL in the ER showed a positive correlation with the decrease in frequency of irreversible ultrastructural changes in the plasma membrane. Therefore, it seemed that direct interactions between the plasma membrane and organelle membranes (and/or plasma membrane) are restricted by the localization of MPL in the ER between biomembranes. On the other hand, MPL formation in the ER was not observed in cold-acclimated leaves of *Arabidopsis*, and only a single or a few layers of ER lamellae (incomplete form) were observed. Thus, understanding the molecular mechanism of MPL function is an interesting approach to increase the potential for MPL formation for the development of high freezing resistance. Also, further experiments may be required to obtain physiological and biochemical information regarding specific features of MPL of the ER.

8.4.2 Deep Supercooling

In xylem tissues of boreal trees, XRPCs are not dehydrated despite the freezing of apoplast water at relatively high subzero temperatures. Therefore, freezable water remaining inside XRPCs is maintained in a supercooled state at

subzero temperatures. Because supercooled water inside xylem cells is retained for longer periods and/or at low subzero temperatures, the supercooling of intracellular water in XRPCs is referred to as deep supercooling (Fig. 8.2). However, the potential for deep supercooling in XRPCs is limited; intracellular freezing may occur as a result of excessive cooling. Therefore, the deep supercooling potential of XRPCs can be evaluated as the breakdown point of supercooling water by detection of an exotherm peak due to latent heat release at relatively low subzero temperatures (low-temperature exotherm, LTE) by differential thermal analysis (DTA) only if the water content of living cells in xylem tissues is sufficient to allow detection (Quamme et al. 1972; George et al. 1982). If not, the limit of deep supercooling of XRPCs cannot be detected by DTA because of undetectable LTEs. At such instances, the curve of thermal response is indistinguishable from that of extracellular freezing in bark cells, which undergo gradual dehydration as the temperature declines. Thus, techniques using cryo-scanning electron microscopy (cryo-SEM) have been developed to determine whether or not intracellular supercooled water is frozen (Fujikawa and Kuroda 2000; Kuroda et al. 2003; Fujikawa 2016).

Cryo-SEM analysis revealed that supercooling of intracellular water in XRPCs in hardwood and softwood species was maintained at −20 to −40 °C in XRPCs without deformation, which indicates the absence of dehydration in these cells (Fujikawa et al. 2009). In addition, during deep supercooling in XRPCs with high freezing resistance below −40 °C, cells shrink as a consequence of incomplete dehydration (Gusta et al. 1983; Kuroda et al. 2003). It seems that the contraction of intracellular solutes because of incomplete dehydration might contribute to a decline in intracellular freezing temperature by freezing-point depression. However, the exact mechanism underlying incomplete dehydration of XRPCs below −40 °C is still unknown. Deep supercooling potential of XRPCs may be associated with the freezing resistance of woody trunks, and latitudinal distribution of trees in cold climate regions is well correlated with the deep supercooling potential of XRPCs (George et al. 1974; Fujikawa and Kuroda 2000).

In deep supercooling XRPCs, intracellular supercooled water in XRPC appears as a tiny water droplet isolated from extracellular ice crystals and can undergo supercooling to the temperature limit of homogeneous ice nucleation (Ashworth and Abeles 1984). Deep supercooling potential, which is evaluated by LTE in DTA, was influenced by modification of xylem tissues using cell wall digestion enzymes or EGTA. Peaks of LTE were shifted to higher temperatures and/or lower peak areas. In particular, pit membrane structure of the cell wall of XRPCs close to vessel may be important for isolating intracellular water from extracellular ice (Wisniewski 1995; Wisniewski et al. 2014). On the other hand, in DTA of Japanese beech (*Fagus crenata*) xylem tissues whose intracellular components of XRPCs were removed by washing with Milli-Q water after repeated freeze-thawing with liquid nitrogen, LTE peaks were detected at around −20 °C in summer and winter tissues, suggesting that water inside cell wall structure can supercool to around −20 °C in Japanese beech xylem in both seasons (Kasuga et al. 2013). Because the supercooling potential of living xylem tissues of Japanese beech is around −40 °C in winter, it is considered that the potential of supercooling of cytoplasmic components in winter was estimated to the difference in the potential between living XRPCs and their cell wall structures. It is possible that seasonal changes in supercooling capability of XRPCs may be associated with cellular factors. However, cell wall components and features were also altered during cold acclimation as mentioned in the previous section. Further analysis is necessary to reveal the physiological importance of cell wall structure on deep supercooling potential in winter. Also, accumulation of compatible solutes such as soluble sugars in XRPCs can affect freezing-point depression of intracellular water. Furthermore, recent studies have shown that some polyphenols, such as flavonol glycosides (Kasuga et al. 2008) and hydrolyzable tannins (Wang et al. 2012), have anti-ice nucleation (supercooling-promoting) activity in solutions,

in the presence of ice-nucleating agents such as bacteria and/or silver iodide. Supercooling activities, which are defined as the differences between freezing temperature of solutions with ice-nucleating agents and those without, ranged from 1 to 10 °C and depended on the types of agent. In some cases, supercooling activities were shown not only as depression in the freezing temperatures but also as decrease in the frequency of freezing at constant subzero temperatures. Polyphenol activity may contribute to the maintenance of supercooled state of intracellular water in XRPCs during winter (Kasuga et al. 2008; Wang et al. 2012). It was also suggested that anti-ice nucleation activity was detected for many polyphenols including hydrolyzable tannins and flavonoid glycosides (Kuwabara et al. 2011, 2012). Although many physiological events such as sugar accumulation (Kasuga et al. 2007), protein composition (Arora and Wisniewski 1994), and gene expression (Takata et al. 2007) in xylem tissues are induced during cold acclimation, the relationship between these physiological factors and the deep supercooling potential of XRPCs requires further investigation.

8.4.3 Extraorgan Freezing of Winter Buds

In the dormant buds of certain coniferous trees, accumulation of extracellular ice crystal masses was not observed in primordial tissues and was observed in specific areas, such as extracellular spaces in basal areas of scales and/or those beneath crown tissue, which localizes below the shoot primordium in a dormant bud (Sakai and Larcher 1987; Ashworth et al. 1989). This freezing behavior of dormant buds of these trees is known as extraorgan freezing (Sakai 1982; Fujikawa 2016) (Fig. 8.3).

Cryo-SEM observations indicated that freezing behaviors are different among tissue cells in dormant buds with potential of extraorgan freezing in larch. In whole dormant buds of larch twigs that were frozen at a slow cooling rate, they survived freezing at −30 °C, and shoot primordial cells underwent incomplete dehydration at subzero temperatures, and remaining freezable water in these cells was supercooled (Endoh et al. 2009). Also, no extracellular ice accumulation was observed in primordial tissues. Conversely, cells of scale and crown tissues responded by extracellular freezing and masses of extracellular ice crystals accumulated in extracellular spaces adjacent to these tissues (Endoh et al. 2009). These specific spaces for extracellular ice accumulation in an extraorgan freezing bud are called an “ice sink” (Ishikawa et al. 2015). Cells surrounding ice sinks have relatively thick cell walls compared to primordial cells with primary cell walls. Freezing resistance of scales and crown tissues, which are the sites for ice sinks, is higher than that of whole dormant buds (Endoh et al. 2014).

When shoot primordial tissues isolated from whole dormant buds of larch were frozen to −30 °C at a slow cooling rate, intracellular freezing was observed in almost half of the cells, and extracellular freezing with severe deformation was also observed in some cells, resulting in lethal injury of the isolated tissues (Endoh et al. 2014). Thus, primordial tissue cells are sensitive to extracellular ice, and primordial cells are protected from extracellular ice by extraorgan freezing in whole dormant buds; therefore, a whole dormant bud functions systematically during extraorgan freezing. Cryo-SEM observation also showed that shoot primordial tissue underwent incomplete dehydration with lack of extracellular void spaces within the tissue during extraorgan freezing in a whole dormant bud of larch. Therefore, it seemed that water was dehydrated from a whole shoot primordial tissue and moved to the ice sink to form masses of extraorgan ices. In extraorgan freezing of a whole dormant bud, sensitivity of primordial cells to extracellular ice is an important factor affecting freezing behavior (Endoh et al. 2014). Therefore, it is believed that structures and responses of the cell wall and plasma membrane to subzero temperatures may influence cell survival in extraorgan freezing conditions of primordial tissues because these structures may function as barriers to extracellular ice in winter generally. Further characterization is

necessary to understand the molecular mechanism of extraorgan freezing of whole dormant buds, including extracellular ice formation in ice sinks and characterization of cell wall and plasma membrane structures.

8.5 Conclusion

Global climate change models predict increases in the mean air temperature and the frequency and severity of meteorological events. Because the ambient temperature strongly affects cold acclimation and deacclimation in plants, milder weather during colder seasons (warming between late autumn and early spring) can decrease or delay the attainment of maximum cold hardiness, resulting in severe frost damage to plants. Overwintering is an essential mechanism by which plants, particularly temperate and boreal trees, can survive in their habitats for many years. To forecast the effects of the predicted climate change on the winter survival of trees, the mechanisms of cold acclimation and deacclimation and freezing adaptation must be understood.

Seasonal and daily fluctuations of light and temperature signals induce transitions from active to dormant states and from frost sensitivity to cold hardiness. In this chapter, we have introduced the typical internal mechanisms by which the shortening of the photoperiod and decrease in temperature are perceived and also by which cold hardiness is developed via morphological and physiological changes in cells in representative boreal trees such as poplar, birch, larch, and spruce. Further studies are evidently required to elucidate the detailed molecular mechanism by which trees perceive light and temperature. Overall, the key question is whether the fundamental molecular mechanism is conserved in other boreal softwood and hardwood species. We have also introduced three types of freezing behavior of tree cells. Among them, deep supercooling of xylem ray parenchyma cells and extraorgan freezing of winter buds are particularly observed in tree species. Furthermore, extracellular freezing in the bark cells of boreal trees achieves an extremely high freezing tolerance compared with that in cells of many herbaceous plants. Because the mechanisms of three types of freezing behaviors are different, the role of structural and physiological features of the cells in bark, xylem, and dormant bud in winter on freezing behaviors and freezing resistances in boreal trees is an interesting issue to be solved.

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The Mechanism of Low-Temperature Tolerance in Fish

9

Kiyoshi Soyano and Yuji Mushirobira

Abstract

In this chapter, we cover the life history of fish in low-temperature environments, including their overwintering behavior and the physiological mechanisms by which they maintain life in cold environments, based on research to date. There is relatively little research on low-temperature tolerance of fish, compared with research on this phenomenon in mammals and birds, which are also vertebrates, and the mechanisms in fish have not been fully elucidated. First, we cover the life history of fish that overwinter by entering dormancy or hibernation. Next, we describe the mechanism that controls body temperature in fish that survive low-temperature environments. Finally, we introduce the physiological mechanisms for survival in extremely low-temperature environments, particularly antifreeze proteins.

Keywords

Dormancy · Hibernation · Ectothermic fish · Endothermic fish · Heat exchange · Antifreeze glycoprotein (AFGP) · Antifreeze protein (AFP)

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Abbreviations

ACTH	Adrenocorticotropic hormone
AFGP	Antifreeze glycoprotein
AFP	Antifreeze protein
CDC48	Cell division cycle protein 48
GH	Growth hormone
GTH	Gonadotropic hormone
LDLR	Low density lipoprotein receptor
MO_2	Muscle oxygen consumption
MSH	Melanophore-stimulating hormone
PRL	Prolactin
SERCA	Sarco-endoplasmic reticulum Ca^2+ ATPase
SL	Somatolactin
TH	Thyroid hormone
TSH	Thyroid-stimulating hormone

9.1 Introduction

In fish, which are ectothermic (heterothermic) animals, the temperature of the environment is a major factor controlling phenomena such as growth and breeding because their body temperature is affected by ambient water temperature (Brett 1971; 1979). Fish move in search of a suitable water temperature (Schurmann and Christiansen 1994; Claireaux et al. 1995). Recently, it has been reported that several fishes control their body temperature using a heat

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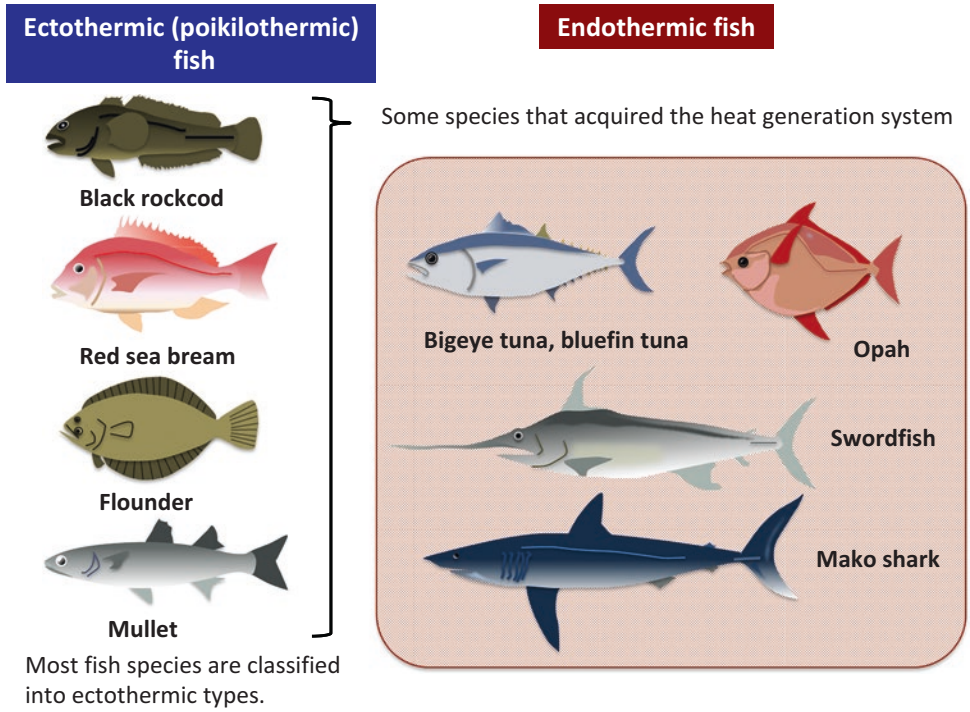

Fig. 9.1 Example of ectothermic and endothermic fish. In fish, there are some species having heat generation system, which is called endothermic fish, although fish is categorized into ectothermic type basically

generation system, in addition to moving to areas with appropriate water temperature. This group of endothermic fishes includes the bigeye tuna *Thunnus obesus* and the opah *Lampris guttatus* (Holland et al. 1992; Wegner et al. 2015) (Fig. 9.1). However, the temperature that they can retain is not high compared with that of homeothermic animals. Furthermore, they lack a heat-radiating mechanism for keeping the body temperature constant, which homeothermic animals possess.

How do fish respond to ambient temperature change? One way is behavioral thermoregulation, in which fish move to an area with suitable water temperature to maintain homeostasis for continuing physiological functions. As the optimum temperature differs for various physiological phases, such as growth and maturation, fish must migrate according to their temperature requirement for each phase in the life cycle.

Migratory fish are able to travel through a wide area, but fishes that have poor swimming ability and inhabit a specific environment are forced to adapt to the ambient temperature, even if the temperature fluctuation is large. These species respond to adverse conditions (i.e., when the water temperature deviates from the appropriate range) by reducing their physiological activity as much as possible. Especially in areas with cold water, fish generally cease physiological activity during the winter season, a condition that is extremely close to the state of hibernation.

Meanwhile, fishes living in environments where the water temperature is low throughout the year, such as the polar zone, have physiological mechanisms for adapting to low water temperatures. Some of these adaptations include the synthesis of an antifreeze protein (AFP) and antifreeze glycoprotein (AFGP) (Harding et al. 2003; DeVries and Cheng 2005), formation of tubulin that can be synthesized at low temperature (Guderley 2004), and lack of hemoglobin (Hemmingsen 1991).

This chapter will explain adaptations to low water temperatures in fish, focusing on three topics: hibernation, body temperature control, and

the mechanism of tolerance to low water temperatures.

9.2 Dormancy of Fish at Low Temperatures

It is difficult to define hibernation in fish. Hibernation refers to a low metabolic state that animals enter under a low-temperature environment, during which they reduce their basal metabolism and consume less energy. This adaptation to winter is well known in mammals (see Chap. 3). Hibernation is characterized by maintenance of an extremely low metabolic state with unusual physiological conditions such as low breathing, low heart rate, low body temperature, etc. It is necessary to have a mechanism to maintain life even at low temperature. However, the condition is not considered hibernation if the physiological conditions are maintained in an active state like sleep, even if the animals have temporarily stopped active behavior and their metabolic activity is suppressed for a long time. These conditions, which are often observed in fish during the winter season, are considered low-temperature dormancy or winter dormancy (Fig. 9.2). Interestingly, some species of fish, such as the Japanese sandeel, enter dormancy during the summer season, when it is called aestivation (Tomiyama and Yanagibashi 2004). As the sleeping state has been observed in fish not only in winter but in summer, the condition is typically considered dormancy. However, recently fish displaying characteristics that are similar to hibernation have been observed (Campbell et al. 2008).

As described above, fish are classified as heterothermic animals whose body temperature depends on the ambient water temperature, except for some fish species that produce heat by themselves (endothermic fish). Therefore, during

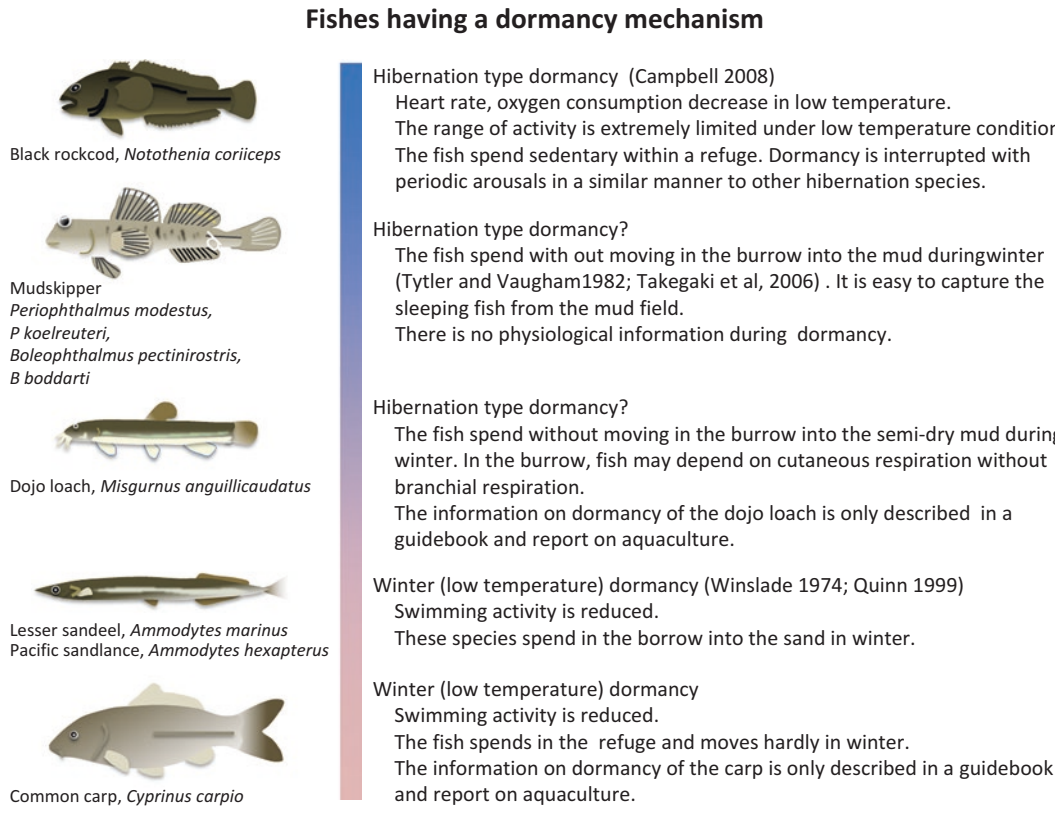

Fig. 9.2 Summarization of dormancy of fish

periods when the water temperature is low, many fish species move to areas with more suitable temperature where they can maintain normal activities. Other fish species enter a low-temperature dormancy during the winter season. The physiological state of the fish during this low-temperature dormancy, as well as the mechanism causing the dormancy, differs from hibernation in mammals, which are homeothermic animals.

The Pacific sandlance *Ammodytes hexapterus* (Quinn 1999), the lesser sandeel *A. marinus* (Winslade 1974), and the black rock cod *Notothenia coriiceps*, inhabiting the Antarctic (Campbell et al. 2008), are fish that reduce their physiological activity in the winter season. Although it is not described sufficiently in the scientific literature, the phenomenon of low-temperature dormancy is known also in the dojo loach *Misgurnus anguillicaudatus* and the mudskippers *Periophthalmus modestus* and *Boleophthalmus pectinirostris*, which escape by burrowing under sediment in the winter season. However, the strategy for entering a low metabolic state varies by species.

The lesser sandeel, inhabiting the North Sea, burrows into sand during winter when the water temperature is low (Winslade 1974). In the coastal areas of the UK, this burrowing behavior is observed from January, after spawning, until April. Among fish reared in different water temperatures (5 °C, 10 °C, and 15 °C), swimming activity was reduced at 5 °C, although activity levels of fish at 10 °C and 15 °C remained high (Winslade 1974). The burrowing behavior appeared to be a response to decreased water temperature. In addition, the burrowing may be related to fat stores, which are probably at their lowest level after spawning. Burrowing as an overwintering strategy appears to be an effective way to retain energy lost during spawning and also reduces vulnerability to predation. The phenomenon may be regarded as an adaptation to survive a period of unsuitable environment in the fishes' life cycle.

N. coriiceps is a teleost that inhabits the Antarctic (Hubold 1991; Knox 2006) and has antifreeze proteins to prevent the freezing of body fluids, even when the water temperature falls below the freezing point (see Sect. 9.5). Although the annual changes in temperature in the Antarctic marine environment are small and the environment is considered thermally stable, this species also reduced its activity levels during the winter season to save metabolic energy (Campbell et al. 2008). From May to November, when the water temperature decreases rapidly to around −2 °C, the growth rate in *N. coriiceps* is sharply suppressed and the heart rate (fH) also decreases. The heart rate is positively correlated with oxygen consumption (MO_2), and it was found that MO_2 also decreases during the period of low water temperature. Campbell et al. (2008) also reported interesting results from behavior tracing of fish, using a static hydrophone array throughout the year.

N. coriiceps had a wide range of activity during the summer season (from December to May). However, from June to August, the low-temperature period, the range of activity was extremely limited. A scuba diver observed that *N. coriiceps* found at 18 m depth, in water that was −1.8 °C, was not able to move and indicated no response even if the diver was holding the fish. Dormancy in *N. coriiceps* involves the active suppression of MO_2 and fH irrespective of temperature, suggesting that some other cue factor initiates dormancy rather than temperature, such as reduction of light in winter. These changes induce a reduction in the growth rate, as reported in other Antarctic notothenioids (Coggan 1997). Dormancy in *N. coriiceps* is distinct from the dormancy observed in temperate fish. The degree of physiological suppression in this fish is similar to that of hibernating animals. Therefore, dormancy in *N. coriiceps* is considered to be hibernation.

The Japanese mudskippers *P. modestus* and *B. pectinirostris* enter dormancy during a low-temperature phase. These species have the ability to breathe air and can move on the surface of a muddy tidal flat using their pectoral fin (Pace 2017). They burrow into the mud to nest and spawn in the burrow (Martin and Ishimatsu 2017). Their behavior and spawning depend on water temperature, with an active period from

spring to early winter. However, in winter when the temperature drops, the mudskipper escapes into a mud burrow. We observed that *P. modestus* disappeared from the surface of tidal flats in late November, after which it did not leave the mud burrow. The burrow in the tidal mud remains approximately 3 °C warmer than the surface of the tidal flat, which is exposed to the outside air and experiences low temperatures in the winter (Soyano et al. unpublished data). In Ariake Sound, Japan, the mud temperature remained between 5 and 8 °C at a depth of 30 cm during the coldest season (Takegaki et al. 2006). When we excavated a mud burrow in February, the time of year when the ambient temperature was lowest, the dormant mudskipper was easily captured from the burrow. The fish was a state of dormancy just after capture, although it awakened and moved a short time later when the ambient temperature was lower than that of the mud burrow.

The fH and MO_2 in *N. coriiceps* are reduced during hibernation (Campbell et al. 2008). Unfortunately there are no data about heart rate and body temperature in the mudskipper before and after winter dormancy, but fH and MO_2 is expected to decrease during winter dormancy in this species. The mechanism appears to be similar as in hibernation. However, *B. pectinirostris* often dies during hibernation. In a rearing experiment that explored tolerance to low temperatures, most individuals of this species died within 24 h at 3 °C and within 15 days at 7 °C under continuous low-temperature conditions (Takegaki et al. 2006). This outcome indicates that the temperature tolerance limit of the fish was exceeded.

Another mudskipper species *P. koelreuteri* and *B. boddarti* inhabiting Kuwait enters dormancy when the ambient temperature falls below 10 °C in winter, while the fish maintains high activity levels at 14–35 °C. This species also remains in the burrow during cold periods to prevent loss of body temperature (Tytler and Vaughan 1982). The mudskipper also appears to use the burrow to reduce the risk of predation associated with the suppression of behavior accompanying low metabolism.

9.3 Thermoregulation in Fish

Thermoregulation in ectothermic animals, including most species of fish, depends on the environmental temperature. Ectothermic fish regulate their body temperature by moving to an area with appropriate water temperature to maintain homeostasis and continue normal physiological function, which is known as behavioral thermoregulation. In contrast, fish that generate heat using a physiological thermoregulation mechanism and maintain a body temperature that is higher than that of the ambient water temperature are termed endothermic fish (Holland and Sibert 1994; Nakamura et al. 2015; Wegner et al. 2015). This ability enhances their ability to engage in feeding and swimming behavior and increases their physiological activity. However, fish with this ability constitute fewer than 0.1% of fish species. Moreover, the thermogenic system in fish can warm only a limited part of the body, whereas mammals are completely endothermic animals. Thus, fish with this ability are called regional endotherms (Wegner et al. 2015). They include the swordfish *Xiphias gladius* (Carey and Robinson 1981), the Atlantic bluefin tuna *Thunnus thynnus* (Block et al. 2001), the Pacific bluefin tuna *T. orientalis* (Kitagawa et al. 2006), the bigeye tuna *T. obesus* (Holland and Sibert 1994), the mako shark *Isurus oxyrinchus* (Bernal et al. 2001), and other large pelagic predatory fish that dive into deep water to find food. For example, mean body temperature is maintained at 4 °C above the ambient water temperature in the blue shark *Prionace glauca*, because the rate of warming in the body is higher than the rate of cooling due to the environmental water (Carey and Scharold 1990).

In the bluefin tuna and the skipjack tuna, thermoregulation is carried out by passing oxygenated blood from the gills into the counter-current vascular retia, warm venous blood vessels that go to the heart from the swimming muscles (Carey and Lawson 1973; Stevens et al. 1974, 2000). According to a study that measured the temperature of red muscle, white muscle, and

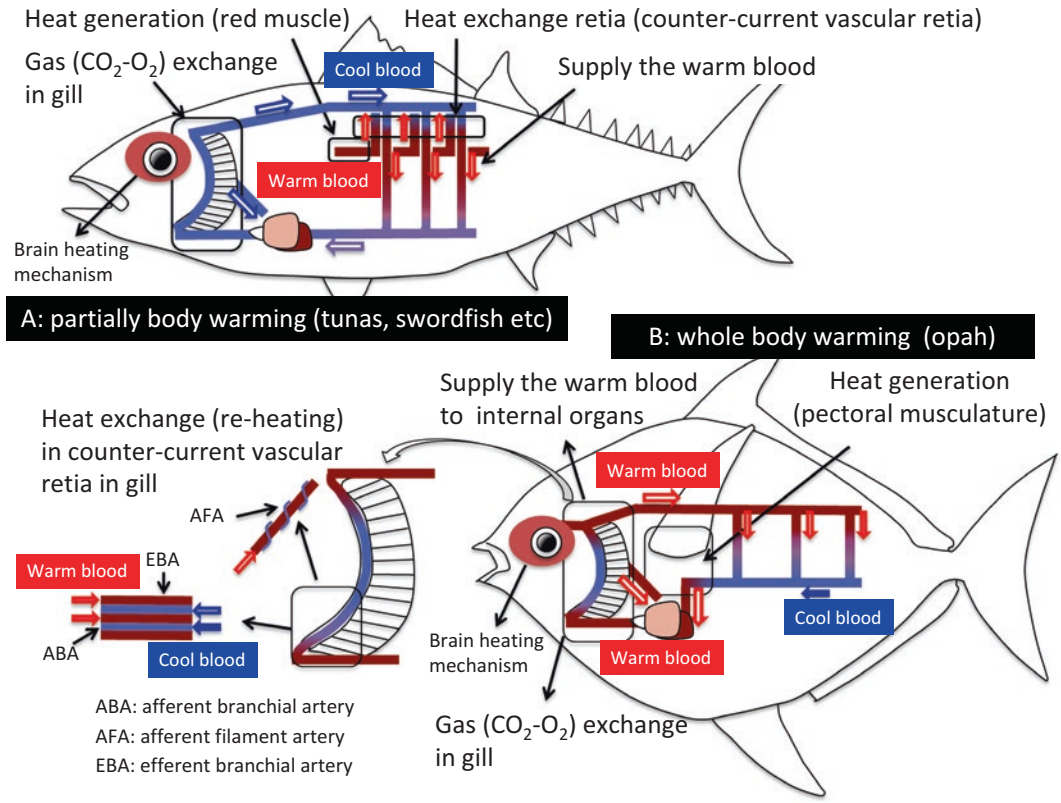

Fig. 9.3 Heat generation and exchange system in fish. There are two type of body warming in fish. (**a**) Partially body warming. The bluefin tuna and swordfish are known as this type, which utilize the red muscle as heat generator. (**b**) Whole body warming. The opah is known as this type, which has the characteristic structure in gill and pectoral musculature for heat generation and exchange

the stomach in the mako shark *I. oxyrinchus*, body temperature is higher than ambient temperature (Bernal et al. 2001). This study showed that the shark has vascular networks (retia mirabilia) that act as counter-current heat exchangers, allowing metabolic heat retention in certain regions of the body, and the mechanism to regulate heat transfer is similar functionally and morphologically to that in tuna (Bernal et al. 2001) (Fig. 9.3). Such partial elevation of body temperature improves temperature-sensitive physiological processes such as a digestion, metabolism, nervous system function, and locomotion (Graham and Dickson 2001).

Interestingly, fish can use temperature control to enhance the function of the eyes and brain. Swordfish have a particularly high ability to use their heating function to increase the temperature of the brain and eyes (Fritsches et al. 2005). This mechanism is fundamentally different from that found in the tuna. The eyes of ectothermic fishes are the same temperature as the surrounding environmental water, so it is expected that the vision potential in the eye will be diminished when the eye temperature decreases due to entering a low-temperature zone. Endothermic open-ocean predators have the ability to warm the retinal area to maintain visual function. The retinal warming in the eye prevents a decline in visual resolution due to the drop in water temperature caused by locomotion to a deep-sea area, thereby helping the predator to capture prey.

As described above, the bluefin tuna and mako shark merely enhance their vision and movement potential temporarily by partially increasing the body temperature. However, the opah *L. guttatus*

is able to warm its whole body by introducing a special heat exchange system (Wegner et al. 2015) (Fig. 9.3). This ability is related to the vascular structure around the heart from the gills. The opah produces heat by flapping its winglike pectoral fins. The warmed blood is sent from the afferent filament artery to the gill via the heart. However, the deoxygenated blood warmed by the flapping of pectoral fins loses heat when the blood undergoes gas exchange at the surface before entering the efferent branchial arteries. The afferent and efferent arteries filament are closely coupled and stacked in an alternating pattern within the gill arch. This structure is very important to rewarm the oxygenated blood after it has cooled in the surfaces of the gill filament. The warmed blood is delivered to the whole body. By employing such a heat exchanging system, the opah can keep its body temperature several degrees higher than the external water temperature. Although this mechanism is different from the low-temperature tolerance of fish inhabiting polar regions, it is considered an important physiological mechanism for adapting to low water temperatures that are experienced on a daily basis.

9.4 Use of Antifreeze Protein to Adapt to Low-Temperature Environment

In the Arctic and Antarctic regions, the water temperature can drop below zero due to supercooling. Fishes in these regions use antifreeze mechanisms to adapt to the extreme temperatures. The plasma freezing point of the bald notothen *Trematomus borchgrevinki* inhabiting the Antarctic Ocean is −2.75 °C, whereas in the black perch *Embiotoca jacksoni*, which is distributed in the temperate zone, it is −0.7 °C. Therefore, body fluid in some fishes does not freeze even if the water temperature drops below zero (DeVries 1982). The mechanism for this phenomenon is antifreeze protein. Glycoproteins that enable a lower plasma freezing point have been isolated from the plasma of fish belonging to the Notothenioidei suborder inhabiting the Antarctic Ocean, and several proteins that can reduce the plasma freezing point are found in other species of fish (Harding et al. 2003). These proteins, called antifreeze glycoproteins (AFGPs) and antifreeze proteins (AFPs), inhibit the growth of ice crystals in plasma by covering the water-accessible surface of ice, resulting in a lower freezing point for plasma and enabling polar fish to survive in seawater below the freezing point.

9.4.1 Characteristics of Antifreeze Proteins

Antifreeze proteins found in fish have been classified in a single class of AFGP and four classes of AFPs (types I–IV). AFGP, a glycoprotein with a molecular weight of 2.6–33 kDa, consists of a number of repeating units of alanine-alanine-threonine and has a side chain of threonine modified with disaccharide, which is involved in binding to ice crystals (Table 9.1). A total of eight AFGPs with different molecular weights were purified from a single fish species and were classified roughly into two types, high molecular (AFGP1–5) and low molecular (AFGP6–8) types (Harding et al. 2003). These proteins have been isolated in Notothenioidei and Gadidae living in cold water (DeVries 1982; Burcham et al. 1984).

Type I AFP is a monomeric protein with an α-helical folded structure and a molecular weight of 3.3–4.4 kDa. The protein consists of a large amount of alanine, threonine, and aspartic acid (Duman and DeVries 1976). AFP was isolated from the winter flounder *Pseudopleuronectes americanus*, shorthorn sculpin *Myoxocephalus scorpius*, and other species, and multiple molecules were purified (Duman and DeVries 1976; Hew et al. 1980). Type 1 AFP is classified into two types, the liver type and the skin type (Gong et al. 1996; Low et al. 1998). Whereas the liver type is a secreted protein, the skin type has no signal peptide and is considered to function intracellularly. Hyperactive AFP, which has an activity level 10–100-fold higher than that of the conventional type I AFP, was isolated from the plasma of winter flounder (Marshall et al. 2005). The molecular weight of this novel AFP was

Table 9.1 Characteristics of antifreeze glycoproteins and antifreeze proteins isolated from fish

	Type	Subclass	Structure	Isolated species
Antifreeze glycoprotein (AFGP)		High molecular type (AFGP1-5)	Repeating units of (Ala-Ala-Thr)n	Antarctic notothenioids (DeVries 1982)
		Low molecular type (APGP6-8)	Disaccharide joined to the Thr	Northern cods (Burcham et al. 1984)
Antifreeze protein (AFP)	Type I	Liver type	Alanine-rich, α-helix	Winter flounder (Duman and DeVries 1976)
				Shorthorn sculpin (Hew et al. 1980)
		Skin type	Alanine-rich, α-helix	Winter flounder (Gong et al. 1996)
			Lack of signal peptide	Shorthorn sculpin (Low et al. 1998)
		Hyperactive type	Alanine-rich, α-helix	Winter flounder (Marshall et al. 2005)
	Type II	Ca2+-dependent	Globular protein	Rainbow smelt (Ewart et al. 1992)
		Ca2+-independent	Five disulfide bonds	Atlantic herring (Liu et al. 2007)
				Sea raven (Slaughter et al. 1981)
				Longsnout poacher (Nishimiya et al. 2008)
	Type III	QAE-binding	Small globular protein	Eelpout (Ko et al. 2003)
		SP- (or CM-) binding		Ocean pout (Hew et al. 1984)
	Type IV	–	Glutamine-rich	Longhorn sculpin (Deng et al. 1997)
			Four-helix bundle	

16,683 Da, and 60% or more of its amino acid composition was alanine, as in the conventional type. The novel AFP is a long rod-like structure with dimeric α-helix.

Type II AFPs are globular proteins with a molecular weight of 11–24 kDa. They have a folding structure consisting of two helices and nine β-strands in two β-sheets, with five disulfide bonds (Gronwald et al. 1998). These proteins are divided into two types, calcium (Ca^{2+})-dependent and calcium-independent (Ewart et al. 1992). The Ca^{2+}-dependent type II AFP was isolated from the smelt *Osmerus mordax* (Ewart et al. 1992) and the Atlantic herring *Clupea harengus* (Liu et al. 2007). This type of protein was purified from the Japanese smelt *Hypomesus nipponensis*. Its function in the body appears to be enabling biological activity to continue in the absence of Ca^{2+} (Yamashita et al. 2003). The Ca^{2+}-independent type has been isolated from the sea raven *Hemitripterus americanus* (Slaughter et al. 1981), the longsnout poacher *Brachyosis rostratus* (Nishimiya et al. 2008), and others. This type of protein has no Ca^{2+} binding sites in the sea raven (Ewart et al. 1992).

Type III AFPs are globular proteins with a molecular weight of 6–7 kDa and a folding structure that includes an α-helix, three 3_{10}-helices, and two β-strands (Choi et al. 2015). These proteins do not contain abundant alanine nor high levels of half-cysteine residues in the primary structure (Hew et al. 1984), and there is an ice-binding site in the C-terminal part (Sönnichsen et al. 1996). The globular protein purified from the Antarctic eelpout *Lycodichthys dearborni* and the ocean pout *Macrozoarces americanus* also belongs to type III AFP, and multiple AFP molecules have been purified from individual fish species (Hew et al. 1984; Ko et al. 2003). These molecules can be divided into the QAE Sephadex binding groups and SP Sephadex (or CM Sephadex) binding groups due to the difference in the binding property with ion exchange carriers (Li et al. 1985).

Type IV AFP has a molecular weight of 12 kDa and has been isolated from the longhorn sculpin *Myoxocephalus octodecimspinosis* (Deng et al. 1997; Deng and Laursen 1998). It has a high number of α-helices and four-helix bundle structures and contains a large amount of glutamine. This protein causes ice crystals to grow as hexagonal trapezohedra, unlike other AFPs.

9.4.2 Acquisition and Molecular Evolution of AFP

AFGP is only found in the Antarctic notothenioid and northern gadid fishes (DeVries 1982; Burcham et al. 1984). Although AFGP of the Antarctic notothenioid is derived from pancreatic trypsinogen, the origin of AFGP in the northern gadid is different from that of the Antarctic notothenioid due to difference in genomic sequence and partial structure, suggesting that AFGP developed in these fishes as a result of convergent evolution (Chen et al. 1997a, b).

Type II AFP is considered to have evolved from pre-existing calcium-dependent C-type lectins because the protein is highly homologous with the sugar chain recognition region of the calcium-dependent lectin (Ewart et al. 1992). In addition, it is possible that type II AFP in certain fish species was acquired by lateral gene transfer (Graham et al. 2012; Sorhannus 2012). For example, although the Atlantic herring and the smelt are systematically separated, the primary structure of AFP is very similar between both species, compared with other orthologous genes.

The sequence of Type III AFP is homologous to the C-terminal region of mammalian sialic acid synthase, suggesting that the synthase is the ancestral protein of Type III AFP (Baardsnes and Davies 2001). Type III AFP is a multicopy gene that is present with approximately 150 copies, many of which are closely linked but irregularly spaced (Hew et al. 1988) in the Newfoundland ocean pout populations. In more southerly population of ocean pout in the New Brunswick, the AFP level is lower, and there are only about one-quarter as many AFP copies. As the gene dosage and the AFP levels show a strong correlation, it appears that low-temperature tolerance was acquired by the multiplicity of genes. The ancestral protein of type I AFP is not well understood. However, it is known that type I AFP shows a multiplicity of genes, similar to type III AFP (Scott et al. 1985; Hew et al. 1988). Thus, species with type I may have acquired low-temperature tolerance by amplification of the AFP gene.

Type IV AFP may have originated in apolipoprotein E3, as this AFP has a similar structure to the LDLR-binding region of apolipoprotein E3 (Deng et al. 1997).

9.4.3 Synthesis and Regulation Mechanism of AFP

AFGP was once thought to be synthesized in the liver and distributed within the circulatory system to prevent the blood from freezing. However, recent research has revealed that the major site of AFGP synthesis in the Antarctic notothenioids is the exocrine pancreas, not the liver (Cheng et al. 2006). AFGP of Arctic cod, acquired as a result of convergent evolution, is also synthesized in the pancreas. The pancreatic AFGP enters the intestinal lumen via the pancreatic duct to prevent ingested ice from nucleating the intestinal fluid. The source of AFGP in plasma is the reabsorbed pancreas-derived AFGP in intestinal fluid. Seasonal changes in plasma AFGP levels have been reported in the saffron cod *Eleginus gracilis* which is in the Arctic cod family and inhabits the northern part of the range, although there are no seasonal changes in AFGP levels in notothenioids inhabiting Antarctic waters, where the annual water temperature hardly changes (Burcham et al. 1984).

Moreover, levels of plasma proteins, including AFGP, were high in the winter, which is thought to increase plasma osmolality, in the saffron cod (Ogawa et al. 1997). The protein levels and osmolality decreased after intraperitoneal injection of salmon prolactin (PRL), suggesting that PRL may act on the kidneys and remove the AFGP from plasma by increasing glomerular filtration. When the glomeruli in the kidneys of saffron cod were observed throughout the year, the

glomeruli in fish collected in winter showed atrophy in comparison with the functional glomeruli in fish collected in summer (Kitagawa et al. 1990). These results indicate that the reduction of glomerular function in association with a decline in AFGP drainage function depends on seasonal changes in glomeruli morphology. Interestingly, the kidneys of Antarctic notothenioids are aglomerular or functionally aglomerular (DeVries 1982; Eastman and DeVries 1986), and the glomerular reduction or aglomerularism are considered to be important mechanisms for conservation of small molecular weight AFGP compounds, vital to living in the polar zone.

As described above, two different forms of type I AFP, the liver type and the skin type, have been isolated from the winter flounder (Gong et al. 1996). Although the liver type is mainly expressed in the liver, the skin type is strongly expressed in the liver and exterior tissues, such as the skin, scales, fin, and gills. Type I AFP in the blood seasonally changes in this species, as does AFGP in the saffron cod (Hew and Fletcher 1979). However, AFP levels in plasma dropped when winter flounder were exposed to pituitary extract, including growth hormone (GH) fraction, even though AFGP of the saffron cod was reduced by PRL treatment in winter (Idler et al. 1989). Moreover, the hypophysectomized winter flounder retained high levels of AFP in plasma (Hew and Fletcher 1979), suggesting that AFP synthesis was suppressed by substances in the pituitary, including GH. As there was no seasonal change in the structure of the kidney in the winter flounder (Boyd and DeVries 1983), it is speculated that the transcription of AFP is promoted due to the reduced secretion of GH in the winter; in consequence, tolerance to low temperatures increases.

Although AFP II, III, and IV are mainly expressed in the liver, their expression is also seen in the pancreas (Cheng et al. 2006). However, there is little information about the mechanism that regulates these AFP types, unlike AFGP and type I AFP. Moreover, it appears that type IV AFP lacks the ability to prevent the blood from freezing on its own because its levels are too low in the blood (Gauthier et al. 2008). As type IV AFP has been detected only in fish that also have type I AFP, it is possible that type IV AFP has other physiological functions besides its role in low-temperature tolerance.

9.5 Biological Factors Related to Low-Temperature Tolerance and Cold Shock

In the previous section, we described the antifreeze proteins and antifreeze glycoproteins that some species utilize to survive extremely low temperatures. A related challenge is surviving a sudden drop in water temperature (cold shock), which induces various physiological changes in fish, including effects on growth, ion regulation, and immune function (Donaldson et al. 2008). Biological factors related to low-temperature tolerance have been reported in association with this response.

9.5.1 The Cell Division Cycle Protein 48 (CDC48)

The cell division cycle protein 48 (CDC48), which is involved in cold tolerance, has been identified, and the gene that codes for it has been cloned (Yamashita et al. 1996; Imamura et al. 2003). The protein is a polypeptide consisting of 806 amino acid residues that promote cell division. CDC48 belongs to the AAA (ATPases associated with diverse cellular activities) ATPase family and is considered an essential factor in cell division and cell cycle progression, as well as playing an important part in cell homeostasis (Moir et al. 1982; Dantuma and Hoppe 2012; Meyer et al. 2012). Its primary function is in the endoplasmic reticulum-associated protein degradation, in which it has a key role in promoting quality control in the degradation (Latterich et al. 1995; Hoppe et al. 2000; Ye et al. 2001; Wolf and Stolz 2012; Gallagher et al. 2014).

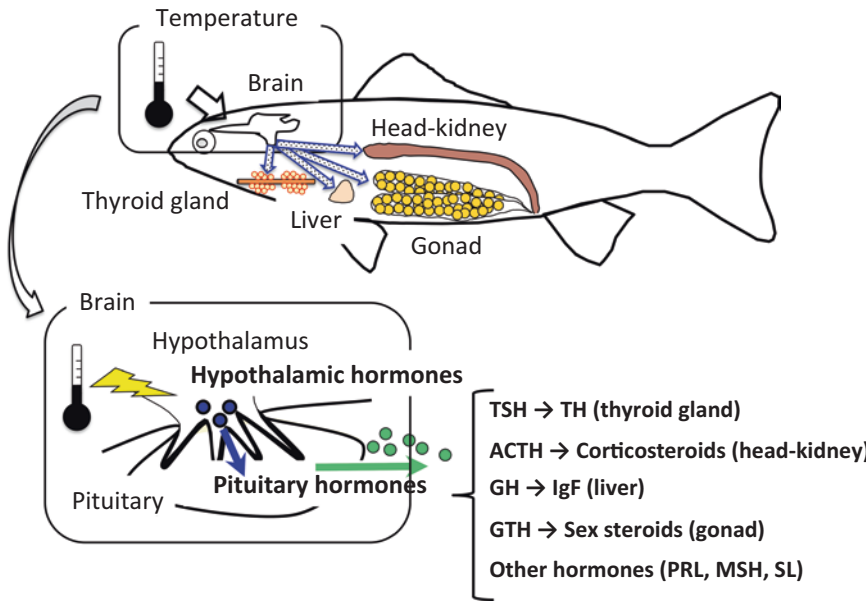

Fig. 9.4 Schematic diagram of the effect of temperature on endocrine system. Water temperature influences on physiological phenomena via hypothalamus-pituitary axis

The *CDC48* gene was isolated from zebrafish, and the effect of temperature on its expression level was investigated using a zebrafish embryo-derived cultured cell line (Imamura et al. 2002, 2003). CDC48 mRNA and protein levels increased as the temperature declined. Interestingly, cell proliferation was enhanced in the cells that overexpressed *CDC48*, which were transfected with cDNA constructs for *CDC48*, under low-temperature condition (Imamura et al. 2003). In addition, expression of this gene increased during the embryonic stage, particularly in the nervous system (Imamura et al. 2012). These findings indicate that the role of CDC48 is degradation of ubiquitinated proteins via activation of ubiquitin-proteasome system function to promote neural development (Imamura et al. 2012). Cold-inducible CDC48 appears to be an important protein with an essential role in controlling cell proliferation and repressing apoptosis in low-temperature conditions in fish.

9.5.2 Hormonal Regulation of Physiological Phenomena in Low-Temperature Conditions

Hormones induce and regulate physiological phenomena in organisms. The synthesis and release of hormones in fish is influenced by ambient water temperature (Fig. 9.4). The brain, the central organ of the nervous system, is also a central part of the endocrine system, and information about the external environment, including temperature, is concentrated in the brain of vertebrates (Crawshaw et al. 1985; Boulant 2000). The hypothalamus is one of the most important parts of the brain for transmitting endocrine information converted from external information. Information about water temperature is also processed in the hypothalamus and is transmitted to the whole body through the endocrine system, centered in the pituitary gland, which is called the hypothalamus-pituitary axis. Hormones

synthesized in the pituitary gland include growth hormone (GH), thyroid-stimulating hormone (TSH), adrenocorticotropic hormone (ACTH), gonadotropic hormone (GTH), prolactin (PRL), melanophore-stimulating hormone (MSH), and somatolactin (SL) (Takei et al. 2016). The secretion and action of these hormones are strongly affected by temperature. In addition, other hormones secreted by stimulation of the pituitary hormone in various organs, including thyroid hormone (TH), sex steroid, and glucocorticoid, are also affected by temperature, directly or indirectly. Although the effect of temperature on the secretion or action of hormones has been investigated in fish, much of the research was conducted in the context of growth, migration, and reproduction in species that are useful for aquaculture and fisheries (Wootton and Smith 2015).

Many studies that examined the relationship between low temperature and hormones have addressed the annual changes in hormone levels related to environmental water temperature and the effect of low temperature on hormone synthesis by temperature manipulation. Unfortunately, there is limited information about the role of hormones in low-temperature tolerance and physiological phenomena at low temperature. TH is known to be a regulator of thermal acclimation in fish (Little et al. 2013). TH has a modulatory function of the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA), a protein associated with muscle and heart function in cold water (Little and Seebacher 2013, 2014). In addition, the heart rate and SERCA activity of fish in which hypothyroidism was induced by propylthiouracil and iopanoic acid were reduced by cold acclimation, while these levels in normal fish acclimated to cold water were high (Little and Seebacher 2013). Moreover, TH treatment in hypothyroid fish restored heart rate and SERCA activity, suggesting that TH plays an important role in maintenance of heart function during cold acclimation.

GH synthesis in fish is modulated by water temperature and is higher during the warmer seasons of the year (Deane and Woo 2009). GH is involved with the process of temperature acclimatization. One of its actions is to control AFP synthesis (Idler et al. 1989). GH synthesis is suppressed during the winter, when AFP levels are high (Fletcher et al. 1989). Moreover, when pituitary extracts including GH were injected in flounder in the wintertime, AFP levels decreased, indicating that GH is one of the regulatory factors of AFP synthesis (Idler et al. 1989).

Cold shock is a stressor that affects various physiological phenomena (Donaldson et al. 2008). The primary response to cold shock is the release of corticosteroids and catecholamines via a neuroendocrine response of the central nervous system (Barton 2002). In tilapia (*Oreochromis aureus*) exposed to cold water, levels of cortisol and catecholamines (epinephrine and norepinephrine) were examined. As a result of acute cold shock, plasma epinephrine, norepinephrine, and cortisol increased with the decreasing water temperature (Chen et al. 2002). These results indicate that cold shock promoted hormone secretion by the hypothalamic-pituitary-adrenal cortical axis. These hormones cause the physiological changes necessary to maintain homeostasis as a secondary response (Barton 2002). However, no further evidence has been obtained indicating the role of cortisol in the low-temperature tolerance of fish.

To understand the mechanism of low-temperature tolerance of fish, it is important to study the role of hormones, including their response to low temperature, because hormones are key factors that mediate the response of organs and cells to environmental conditions.

9.6 Perspectives

As climate change wreaks changes in oceans, rivers, and lakes, it is important to elucidate the mechanism of low-temperature tolerance in fish in order to understand the biological effects of environmental fluctuation and to take necessary measures to conserve aquatic animals. However, there is too little information on the biological responses of fish to low temperatures, which is fundamental for understanding the physiological mechanism of low-temperature tolerance. In addition to gathering these data, it is important to

clarify the mechanisms of low-temperature tolerance, through research on the expression of genes and proteins affected by temperature fluctuation, the functional analysis of these genes and proteins, etc.

Information about the ecological and physiological responses of fish to low temperatures in polar regions is increasing. However, research on this issue should not only target fish inhabiting polar regions. Research should also be conducted on other fish species that show special responses to low temperatures.

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Part II

Adaptation Mechanisms for Desiccation

10 Mechanisms Underlying Freezing and Desiccation Tolerance in Bryophytes

Daisuke Takezawa

Abstract

Bryophytes are small land plants that have many morphological and physiological features different from vascular plants. With distinct water relations of bryophytes, many bryophyte species exhibit high degrees of tolerance to freezing and desiccation. The tolerance is sustained by the constitutive repair mechanism and the inducible mechanism regulated by environmental signals that provoke specific responses within the cells. Bryophyte cells sense changes in environmental conditions such as decreases in osmotic potential and temperature and that some responses are likely to be mediated by the stress hormone, abscisic acid. Due to their simple structures and high degrees of dehydration tolerance, bryophytes are useful for physiological studies on abiotic stress response and also for analysis of signal sensing and transduction of environmental signals. Furthermore, the basal phylogenetic position of bryophytes in land plants provides many insights into the evolutionary events for conquest of land by the ancestors of plants and subsequent diversification of species as well as their survival strategies in the terrestrial environment.

Keywords

Bryophytes · Environmental stress · Desiccation · Freezing · Abscisic acid

Abbreviations

ABA	Abscisic acid
ABRE	ABA-responsive element
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
FW	Fresh weight
GC-MS	Gas chromatography-mass spectroscopy
LEA	Late embryogenesis abundant
MPa	Megapascal
PP2C	Protein phosphatase 2C
PSII	Photosystem II
PYR/PYL/RCAR	Pyrabactin resistance/pyrabactin resistance-like/regulatory component of ABA receptor
RH	Relative humidity
SnRK2	Sucrose non-fermenting1-related kinase2

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10.1 Introduction

Bryophytes are a group of land plants comprising three plant divisions, mosses, liverworts, and hornworts, each with approximately 10,000, 8000, and 400 extant species living on earth (Vanderpoorten and Goffinet 2009). Unlike vascular plants, bryophytes have haploid (gametophyte)-dominant life cycles, and the sizes are generally small. It is thought that bryophytes favor a moist environment such as shady areas in forests, but many species actually inhabit harsh environments including arid and polar regions (Scott 1982; Peat et al. 2006). One of the important features of bryophytes in environmental survival strategies is their body with distinct water relations. In vascular plants, water taken up from roots is transported to shoots by a pressure gradient along the xylem generated by transpiration. The transpiration is tightly controlled by the stoma on the leaf surface, and water loss from other dermal tissues is prevented by a thick cuticle layer. In contrast, water conduction in many bryophytes relies on external capillary action, while limited species have developed internal water-conducting systems resembling the xylem. Bryophytes lack established dermal tissues, and their water potential (vapor pressure) equilibrates with that in the surrounding environment. These features allow the body of bryophytes to undergo rapid dehydration when water potential in the air decreases from nearly zero MPa (100% RH) in a hydrated state to below −100 MPa (less than 50% RH). However, many bryophytes possess a high degree of dehydration tolerance that enables them to survive hyperosmosis, desiccation, and long-term freezing in a state similar to cryptobiosis (Clegg 2001). High levels of tolerance and regeneration capacity allowed survival of mosses and liverworts from samples kept as a herbarium or those preserved in permaforests for a long period of time (Longton and Holdgate 1967; Breuil-Sée 1993).

It has been suggested that some bryophyte species constantly maintain the cellular components necessary for protection of cells against dehydration, enabling desiccation tolerance as a constitutive trait, while other species have mechanisms for induction of these components when they are placed under dehydrating conditions (Oliver et al. 2005). In the latter case, induced accumulation of both low-molecular-weight soluble sugars such as sucrose and hydrophilic proteins similar to late embryogenesis abundant (LEA) proteins for dehydration tolerance appears to be a feature common in tissues in these bryophytes. Soluble sugars and the LEA proteins are the two major components important for tolerance to cellular dehydration in orthodox seeds and vegetative tissues of desiccation-tolerant angiosperms (Ingram and Bartels 1996; Buitink et al. 2002). These two components are thought to protect cell membranes and other macromolecules from damage caused by cellular dehydration. The inducible features in bryophytes for these components enable physiological switching between vigorous growth under favorable conditions and expression of desiccation tolerance under water-limiting conditions for maximum survival in the environment they inhabit.

Freezing is an environmental factor that causes severe dehydration of plant tissues. High freezing tolerance of many bryophyte species has been reported, and the freezing tolerance greatly depends on the environment they inhabit. Some specific bryophyte species dominate flora of frigid regions where few vascular plants inhabit. However, it is known that some cosmopolitan mosses can be also found in the frigid area such as continental Antarctica, indicating a high level of adaptive capacity of bryophytes to different environments. It has been reported that some temperate species of bryophytes undergo seasonal cold acclimation, indicating that these bryophytes can sense a decrease in temperatures in the environment to provoke intracellular mechanisms for developing freezing tolerance, similar to many temperate vascular plant species. However, the response to low temperature under the controlled laboratory conditions has been analyzed only in limited bryophyte species, and little is known about the physiological mechanisms of cold responses. Possible roles of soluble sugars that accumulate during cold acclimation in freezing tolerance have been suggested in some

bryophytes but not in other species, especially those in very cold regions. In angiosperms, a molecular mechanism involving "CBF/DREB regulon" is known to be important for induction of numerous genes including various cold-regulated (*COR*) genes in response to cold (Park et al. 2015). However, the presence of such mechanism has not been demonstrated in bryophytes.

Recent studies have revealed that phytohormone abscisic acid (ABA) plays a role in inducible desiccation and freezing tolerance in bryophytes (Minami et al. 2003a, b). ABA induces expression of genes for LEA-like proteins, enzymes for antioxidant production, and many bryophyte-specific proteins for which functions have not been determined. Genome-wide sequence analysis of the model bryophytes *Physcomitrella patens* and *Marchantia polymorpha* has revealed conservation of genes for ABA biosynthesis, ABA receptors, signaling molecules, and transcription factors for ABA-induced gene expression in bryophytes. With the development of techniques for gene manipulation including genetic transformation, gene targeting, and genome editing, conserved as well as bryophyte-specific mechanisms underlying stress responses mediated and not mediated by ABA are under investigation.

From an evolutionary viewpoint, bryophytes are considered to be the oldest living remnants of the plant ancestor that had colonized the land in mid-Ordovician (Kenrick and Crane 1997; Mischler and Churchil 1984). Land colonization by plants should be achieved through a number of genetic modifications for physiological adaptations in the terrestrial environment. Acquisition of tolerance to dehydration and temperature fluctuations is postulated to be one of the key adaptations for survivals in the terrestrial environment (Oliver et al. 2000a, b; Renzaglia et al. 2000). These modifications include acquisition of mechanisms for sensing environmental water potential and temperatures and provoking expression of genes for production of a range of molecules that protect cells from stress-induced damage. The modifications should also include the recruitment of ABA as a phytohormone for a fine control of balance between growth and stress tolerance, with a constitution of the signaling network connecting ABA and abiotic stress signals (Sakata et al. 2014). Hence, comparative studies on stress responses in bryophytes and other land plants should provide many insights into the evolutionary events required for colonization of land by the ancestors of land plants.

This chapter describes environmental stress-adaptive features characteristic to bryophytes, focusing on their responses to water deficit and low temperature. These features include conserved roles of ABA in sugar and LEA protein accumulation as well as structural modifications of intracellular organelles. High levels of dehydration tolerance that is common in bryophytes but has been lost in most of the vegetative tissues of vascular plants are likely to be an archetypal feature; thus, understanding mechanisms underlying the tolerance would provide us with deep insights into strategies for environmental stress responses in all land plants.

10.2 Desiccation Tolerance of Tissues of Bryophytes

Plant tissues contain 85–90% water on average, and vegetative tissues such as leaf mesophylls of most vascular plants are severely damaged when the cells are exposed to water potentials of −11 to −5.5 MPa, equivalent to 95–96% relative humidity (RH) (Larcher 1995). In contrast, cells of certain plants including bryophytes can survive even when the water potential has decreased to −298 MPa (11% RH) (Bewley 1979). High levels of desiccation tolerance are common in many bryophyte species, while there are only a limited number of vascular plant species belonging to specific phylogenic clades (60–70 species of pteridophytes and approximately 60 species of angiosperms) which are recorded as desiccation tolerant (Bewley 1979; Oliver et al. 2000a, b). Xerophytic features of bryophytes have been known for years (Watson 1914) and have been studied from ecological, physiological, and biochemical viewpoints (Oliver et al. 2005; Toldi et al. 2009). Bryophyte gametophytes are often

described as poikilohydric; they equilibrate their tissue water potential to that of the surroundings. A decrease in water potential in the environment thus leads to rapid water loss from tissues but does not disturb the protoplasmic structure of cells (Proctor and Tuba 2002). Being poikilohydric, most bryophyte species can readily survive moderate levels of desiccation (−20 to −40 MPa), while most crop species can survive only −1.5 to −3 MPa (Proctor and Pence 2002). The desiccation-tolerant moss such as *Tortula ruralis* (*Syntrichia ruralis*) can undergo equilibration with dry air and exhibits tolerance to nearly complete desiccation (Oliver et al. 2000a, b; Wood and Oliver 2004). Proctor (2003) analyzed the survival of 11 desiccation-tolerant bryophytes exposed to drying for periods up to 240 days. Highly desiccation-tolerant species such as *Grimmia pulvinata* and *Tortula ruralis* survived desiccation of −100 to −200 MPa (20–45% RH) for 30–120 days, and moderately tolerant species such as *Porella platyphylla* survived best under −41 MPa (74% RH). Wood (2007) analyzed the desiccation tolerance of 210 bryophyte species and found that 158 species of mosses, 51 species of liverworts, and 1 species of hornwort possess desiccation tolerance in their vegetative tissues.

Physiological aspects of desiccation tolerance in bryophytes have been studied focusing on their capacity of rapid recovery in respiration and photosynthesis following a cycle of dehydration and rehydration. Even after loss of most of the tissue water, desiccation-tolerant bryophyte species quickly recover respiration upon rehydration. The desiccation-tolerant moss *Polytrichum formosum* can recover respiration in a few minutes after rewetting (Proctor et al. 2007). Recovery of the photosystems upon rehydration as indicated by *Fv*/*Fm* (maximum quantum efficiency of photosystem II) is also rapid (Proctor and Smirnoff 2000), suggesting that the integrity of the thylakoid membrane is preserved during desiccation and rehydration. Heber et al. (2000) showed chlorophyll fluorescence in the alpine moss *Grimmia alpestris* that was very low in a dehydrated state and increased upon rehydration. The results are in contrast with results for alpine angiosperms that retain high chlorophyll fluorescence in a dehydration state and suffer from light-induced photodamage. Nabe et al. (2007) showed that desiccation-tolerant moss species such as *Bryum argenteum* and *Hypnum plumaeforme* lost most of their PSII activity (*Fv*/*Fm*) along with a decrease in photosynthesis (*Fm′*—*F*)/*Fm′* upon dehydration. Upon rewetting, both *B. argenteum* and *H. plumaeforme* rapidly recovered both photosynthesis and PSII activity. In contrast, desiccation-sensitive species such as the liverwort *Marchantia polymorpha* and the moss *Philonotis falcata* retained a high level of PSII activity even when photosynthesis was totally inhibited by dehydration, indicating that a photosynthetic burst took place (Nabe et al. 2007).

Although the initial recovery of PSII activity by rewetting is rapid in desiccation-tolerant bryophytes, complete recovery of physiological function might take a longer period. In the moss *Polytrichum formosum*, initial recovery of photosynthesis is so rapid that *Fv*/*Fm* reached 80% of its pre-desiccation value after 10 min of rewetting, but complete recovery of *Fv*/*Fm* took 24 h (Proctor et al. 2007). Li et al. (2014) showed by two-dimensional gel electrophoresis that the thylakoid protein complexes degraded by dehydration reassemble during rehydration, which affects the full recovery of photosynthesis in *B. argenteum*. These results indicate that desiccation-tolerant mosses have a constitutive mechanism for rapid recovery and a rather slow repair mechanism after rewetting, the latter of which might involve the process of de novo protein synthesis (Oliver 1996).

While highly desiccation-tolerant bryophyte species withstand rapid drying, many bryophytes survive better when the rate of drying is slow (Dilks and Proctor 1976; Krochko et al. 1978; Werner et al. 1991). In *Physcomitrella patens*, rapid drying causes membrane damage of protonema cells, but slow drying improves survival and chlorophyll fluorescence after rewetting (Koster et al. 2010; Greenwood and Stark 2014). These bryophytes are thought to have inducible tolerance mechanisms, in which slow dehydration provoked cellular mechanisms for a forthcoming desiccation that causes much more severe damage. These bryophytes are referred as species with “modified”

desiccation tolerance (Krochko et al. 1978; Cruz de Carvalho et al. 2011; Stark et al. 2013). During slow desiccation, species with modified desiccation tolerance undergo structural and physiological modifications by which they produce intracellular environment necessary for desiccation tolerance. A possible role of ABA in enhancement of tolerance during slow desiccation has been suggested (Beckett et al. 2000; Wise and Tunnacliffe 2004) (discussed later).

10.3 Responses to Freezing

It has been described that bryophytes have a certain degree of frost tolerance (Sakai and Larcher 1987). However, the tolerance levels vary much depending on species, natural habitats, tissues, age, and seasons. What might be distinct from vascular plants is that even tropical species, including mosses and liverworts that do not experience freezing temperature in nature, can withstand freezing temperatures of −7 to −14 °C (Biebl 1967). Temperate species appear to be even more tolerant to freezing. Ochi (1952) found that 18 moss species in different habitats were resistant to freezing to −20 °C and that some were resistant to freezing to −27 °C. Hudson and Brustkern (1965) analyzed age-dependent differences in freezing tolerance in young and mature leaves of the moss *Mnium undulatum*. When mature leaves were cooled slowly to a temperature below 0 °C, the tissues underwent extracellular freezing. It was found that even nonhardened leaves can be cooled to −30 °C without injury, and hardened leaves withstood a temperature of −130 °C. In contrast, leaves from a young shoot did not withstand temperatures below −12 °C (Hudson and Brustkern 1965). Rütten and Santarius (1992b) also analyzed age-related differences in frost sensitivity in two *Plagiomnium* mosses and showed that mature leaves are more tolerant to frost than are young leaves. Balagurova et al. (1996) analyzed frost tolerance of five *Sphagnum* mosses species and showed that freezing tolerance ranged from −16.1 to −21.8 °C, with possible correlation with their geographic origin: the least tolerant species originated from a region where the climate is less severe than in the origins of the cold-resistant species. What is characteristic to bryophytes might be the fact that freezing tolerance is greatly affected by their hydration status. Dilks and Proctor (1975) showed that mosses in a dry state could readily withstand a temperature of −30 °C without injury but that most of the species tested were killed by freezing at −10 °C in a moist state. Desiccation-tolerant species generally survive extremely low temperatures in a dry state. Bewley (1973) reported that the shoots of the desiccation-tolerant moss *Tortula ruralis* in a dry state survived even after exposure to liquid nitrogen and that non-desiccated shoots were less tolerant to freezing in liquid nitrogen. Clausen (1964) showed that many liverworts tolerated freezing at −10 °C and that some species tolerated freezing at −40 °C. Even in less tolerant species, frost tolerance was enhanced when tissues were slightly dehydrated by salt or sugar solutions. Thalloid liverworts are generally less tolerant than leafy liverworts to freezing. Dilks and Proctor (1975) also analyzed freezing tolerance of liverwort species and showed that most of the leafy liverworts in a moist state withstood rapid cooling to −5 °C for 6 h. The leafy liverwort *Plagiochila spinulosa* even withstood cooling to −5 °C for periods of 1–2 weeks. In contrast, the thalloid liverworts *Conocephalum conicum*, *Targionia hypophylla*, and *Pellia epiphylla* were killed by cooling to −5 °C.

Ecological studies revealed that bryophytes have capacity to adapt to severe cold environment: many bryophyte species dominate in flora of high latitude area, where only a small number of vascular plants are found (Streere and Inoue 1978; Longton 1988). It has been shown that over 111 species of mosses and 27 species of liverworts dominate in high latitude ecosystems in the Antarctic (Bramley-Alves et al. 2014). Interestingly, leafy liverworts are rare in comparison with thalloid liverworts in the Antarctic (Newsham 2010). Antarctic moss species appear to have photosynthetic characteristics similar to that of constitutively desiccation-tolerant moss species. Freezing caused a reduction in *Fv*/*Fm*, but that was reversed after thawing, indicating

the presence of rapid repair mechanisms of photosynthetic apparatus (Lovelock et al. 1995). Some bryophytes in polar ecosystems tolerate long periods of freezing, possibly by being in a cryptobiotic state. La Farge et al. (2013) showed regrowth of bryophyte tissue buried by ice in Little Ice Age (from the fourteenth to nineteenth centuries), indicating survival for an estimated 400 years in polar ecosystems. The survival and viability of the moss *Chorisodontium aciphyllum* and the liverwort *Cephaloziella* sp. buried deep within an Antarctic moss bank preserved in permafrost for over 1500 years were also shown (Roads et al. 2014). It was also reported that the mosses *Bryum pseudotriquetrum* and *Sanionia uncinata* survived by cryptobiosis for six centuries in a cold-based glacier burial in the Antarctica (Cannone et al. 2017).

10.4 Cryopreservation of Bryophytes

Grimsley and Withers (1983) used dimethyl sulfoxide (DMSO) and glucose for cryopreservation of *P. patens*, and they showed that treatment with 0.5 M mannitol for partial dehydration improved survival after cryopreservation. Christianson (1998) performed cryopreservation of moss species in a DMSO-glucose solution and showed that pretreatment with ABA and proline was effective for better survival after preservation. By using this method, two Sphagnopsida mosses and three Bryopsida mosses were cryopreserved and could also be stored at −80 °C for at least 1 year (Christianson 1998). Yamazaki et al. (2004) reported that pretreatment with ABA and sucrose improved the efficiency of cryopreservation of the moss *Pogonatum inflexum*. By modifying the protocol of Grimsley and Withers (1983) and Christianson (1998) with an optimized freezing and thawing regime, Schulte and Reski (2004) established a high-throughput cryopreservation protocol for numerous mutant lines of *P. patens*. Burch and Wilkinson (2002) reported encapsulation-dehydration of protonemata of the moss *Ditrichum cornubicum* and showed that pretreatment with ABA and sucrose improves cryopreservation. A similar protocol is also used for cryopreservation of various moss species (Rowntree and Ramsay 2005, 2009).

Some desiccation-tolerant moss species might be cryopreserved without any cryoprotectant. Burch (2003) conducted preservation of protonemata of the desiccation-tolerant species *Bryum rubens* and the species *Cyclodictyon laetevirens* and *Ditrichum cornubicum* with limited desiccation tolerance. Protonemata of *B. rubens* survived well after desiccation and cryopreservation with or without encapsulation, while only 30–20% of *D. cornubicum* survived freezing even after encapsulation and *C. laetevirens* did not survive either dehydration or freezing. Segreto et al. (2010) reported that naturally cold-acclimated desiccation-tolerant mosses could be cryopreserved without pretreatment. Furthermore, Yamazaki et al. (2009) reported that spore-derived cell cultures of *Pogonatum inflexum* and *Polytrichum commune* could be cryopreserved after slow desiccation without pretreatment (the culture medium contained 4% sucrose, however). A vitrification procedure for cryopreservation is also applicable to some mosses. Mallon et al. (2001) showed that gametophores, protonemata, and protonemal brood cells of the moss *Splachnum ampullaceum* were cryopreserved by using the plant vitrification solution PVS2, for which exposure to a loading solution containing 2 M glycerol and 0.4 M was a prerequisite for a high survival rate in all samples.

For liverworts, cryopreservation was reported for protoplasts of *M. polymorpha* by slow freezing (Takeuchi et al. 1980). Pence (1998) examined the effectiveness of pretreatment for survival after cryopreservation of liverwort species by encapsulation and dehydration. She showed that ABA pretreatment improved cryopreservation of *Riccia fluitans* but that ABA pretreatment was not effective for survival of *Plagiochila* species. ABA was effective for cryopreservation of *M. polymorpha*, but it was effective but only when the thalli of *M. polymorpha* were encapsulated with 0.75 M sucrose (Pence 1998). Without ABA, partial dehydration might be effective for cryopreservation of *M. polymorpha*. Gemmae of *M. polymorpha* were partially dried with silica gel

for 3 h and then cryopreserved in liquid nitrogen, and the germination rates were 68% after 1-day cryopreservation and 59% after storage for 75 days of cryopreservation (Wu et al. 2015). More recently, Tanaka et al. (2016) reported a protocol for cryopreservation of *M. polymorpha* gemmae by 1-day pretreatment with 0.3 M sucrose and rapid freezing with 2 M glycerol and 1 M sucrose resulted in 100% survival without encapsulation.

10.5 Cellular Components Associated with Stress Tolerance of Bryophytes

10.5.1 Soluble Sugars

Low-molecular-weight soluble sugars generally accumulate upon acquisition of desiccation tolerance in various eukaryotic organisms (Crowe et al. 1992). Sucrose appears to be the common and abundant soluble sugar in bryophytes similar to vascular plants, but the accumulation of hexoses, polyols, fructans, raffinose family oligosaccharides, and trehalose has also been reported (Bewley et al. 1978; Marschall et al. 1998; Melick and Seppelt 1994; Roser et al. 1992; Zúñiga-González et al. 2016). Bewley et al. (1978) examined contents of sucrose, a major soluble sugar, in the desiccation-tolerant moss *Tortula ruralis* and the desiccation-sensitive moss *Cratoneuron filicinum*. However, they found that levels of accumulation of sucrose between these two species were similar and the levels were not affected by slow or rapid desiccation or light and dark conditions. Smirnoff (1992) analyzed sugar contents of shoots of the six moss species and concluded that high sucrose contributes to the desiccation tolerance but that constitutively desiccation-tolerant species do not have inducible mechanisms to accumulate sugars, and its level is maintained high in tissues with or without environmental stress. Antarctic bryophytes such as *Bryum pseudotriquetrum* and *Grimmia antarctici* also showed little seasonal changes in sugar levels (Melick and Seppelt 1994). On the other hand, Rütten and Santarius (1992a, b) analyzed soluble sugar accumulation in winter and the correlation with enhancement of frost tolerance in bryophyte species. In *Polytrichum formosum*, *Atrichum undulatum*, and two *Plagiomnium* species, the amount of sucrose in winter was twofold to threefold higher than that in summer. In contrast, there was no significant difference between the sugar contents in summer and winter in *Mnium hornum* and *Brachythecium rutabulum*, while frost tolerance in winter in those mosses was more than 10 °C greater than that in summer. Slow desiccation also increased the concentration of soluble sugars in the mosses *Atrichum androgynum* and *A. undulatum*, indicating that the sugars might play a role in the inducible desiccation tolerance (Mayaba et al. 2001; Hu et al. 2016).

In the model moss *P. patens*, exogenous ABA increases accumulation of soluble sugars in protonemata, with increases in cellular osmotic concentrations and enhancement of freezing tolerance (Nagao et al. 2005). It was shown that ABA increases tolerance to freezing and desiccation with accumulation of sucrose in *P. patens* (Minami et al. 2003a, b; Oldenhof et al. 2006). However, accumulation of sucrose alone appears to be insufficient for the tolerance because cycloheximide and okadaic acid, both of which reduced ABA-induced freezing tolerance, did not affect accumulation of sucrose (Minami et al. 2003a, b). Nagao et al. (2006) reported that ABA treatment induced accumulation of not only sucrose but also the trisaccharide theanderose, for which cycloheximide inhibited accumulation. The results indicate that ABA-induced de novo synthesis of proteins is necessary for the synthesis of theanderose but not necessary for accumulation of sucrose.

Increased sugar accumulation appears to also be important for the development of stress tolerance in liverworts. Pence et al. (2005) showed that enhancement by ABA of desiccation tolerance is correlated with accumulation of soluble carbohydrate in thalli of the liverworts *Riccia fluitans* and *Pallavicinia lyellii* but not in thalli of *Marchantia polymorpha*. Although thalli of *M. polymorpha* do not accumulate much sucrose, its gemmalings accumulate sucrose in response to ABA, and the accumulation of sucrose is

correlated with enhancement of freezing and desiccation tolerance (Akter et al. 2014). It has also been shown that desiccation tolerance of *M. polymorpha* callus cells can be induced by preculture with 0.5 M sucrose and that accumulation of sucrose in the cells contributed to acceleration of glass transition at a higher temperature (Hatanaka and Sugawara 2010).

10.5.2 LEA-Like Proteins

LEA proteins have been characterized as proteins that accumulate during maturation of orthodox seeds when the tissues develop desiccation tolerance. Several classes of LEA proteins and diverse LEA-like proteins are expressed in vegetative tissues of various angiosperms, especially in response to drought, cold, and salinity stress. Due to their highly hydrophilic nature with specific amino acid repeat motifs, these proteins are postulated to protect cellular structures and macromolecules such as membranes and proteins from dehydration effects (Wise and Tunnacliffe 2004), although the exact functions of various types of LEA-like proteins remain unclear. In bryophytes, the presence of proteins with a highly hydrophilic trait induced by ABA and hyperosmotic mannitol was shown in *P. patens* (Knight et al. 1995). A gene encoding a 30-kDa group 3 LEA-like protein was identified in the amphibious liverwort *Riccia fluitans* as a gene induced by ABA treatment that enhances desiccation tolerance and transition from an aqueous form to land form (Hellwege et al. 1996). A group 3 LEA-like protein was also identified in cultured callus cells of *M. polymorpha* as a protein that was increased with sucrose-induced desiccation tolerance (Hatanaka et al. 2014). On the other hand, Saavedra et al. (2006) reported a role of the protein similar to dehydrin (DHN) that belongs to group 2 LEA proteins in *P. patens*. A gene knockout line of *PpDHN1* showed poor recovery from salt and osmotic stress in comparison with the wild type, indicating that DHNs are required for protection of cells from dehydration stress. Transgenic *Arabidopsis* plants overexpressing two *PpDHN1* genes exhibited enhanced tolerance to drought and salinity (Li et al. 2017). Transcriptome analysis indicated that many of *LEA*-like genes have been identified in *P. patens* and *M. polymorpha*, and their expression is typically increased by ABA, cold, and hyperosmosis (See Sect. 10.7). Some LEA-like proteins might play a role in protection of cells from damage caused by rehydration after desiccation. Oliver et al. (2004) analyzed the "rehydration transcriptome" in the desiccation-tolerant moss *Tortula ruralis* and showed that the moss expresses abundant transcripts for LEA and LEA-like proteins, which might protect cells from injury during rehydration.

10.6 Cytological Changes

Studies on vegetative tissues of bryophytes have revealed that characteristic structural changes in intracellular organelles undergo during dehydration and rehydration. These structural changes are observed in both mosses and liverworts, with some characteristics resembling those reported in desiccation-tolerant vascular plants. The changes are typically found in morphology of vacuoles, chloroplasts, mitochondria, and cytoskeleton, in association with their drastic spatial rearrangement within the cell (Nagao et al. 2005; Proctor et al. 2007; Pressel et al. 2009). Cytological analysis of the desiccation-tolerant moss *Tortula ruralis* indicated that desiccation and rehydration do not disrupt membrane structures of cellular organelles (Platt et al. 1994). In the leptoid (food-conducting cell) and meristematic cells in the moss *Polytrichum formosum*, although desiccation had no obvious damage to the cells, there were changes in shapes of the plastids and mitochondria and structural modifications in the endomembrane domains and microtubular cytoskeleton (Pressel et al. 2006). Proctor et al. (2007) observed that thylakoids, grana, and mitochondrial cristae of chlorenchyma cells in *P. formosum* remained intact throughout the drying-rewetting cycle, but there were some changes in lobes and lamellar extensions in chloroplasts, which were obvious in the normally hydrated state but rounded off upon desiccation.

After rehydration, it took 24 h before recovery of these membrane structures, concomitant with full recovery of photosynthesis (Proctor et al. 2007). These features were also found in the cells of the desiccation-tolerant lycophyte *Selaginella lepidophylla* during desiccation and rehydration (Platt et al. 1994).

Cytological studies also indicated that numerous small vacuoles are observed in the periphery in the meristematic cells, leptoids, and leaf lamella cells in *P. formosum* that was undergoing dehydration (Platt et al. 1994; Proctor et al. 2007). Fragmented vacuoles are also found in gametophore cells of *P. patens* undergoing gradual desiccation (Wang et al. 2009a, b). Nagao et al. (2005) reported that treatment with ABA induces formation of fragmented vacuoles in protonema cells of *P. patens*, indicating that ABA mediates dehydration-induced vacuolar fragmentation. Small vacuoles are typically observed in the resurrection vascular plants such as *Craterostigma wilmsii* and *Sporobolus stapfianus* in a desiccated state (Quartacci et al. 1997; Farrant 2000), as well as in plant cells undergoing cold acclimation (Siminovitch et al. 1975). How these cytological changes influence dehydration tolerance is yet to be clarified. Granted membrane instability is one of the major causes of dehydration-induced injury of cells, above changes can be assumed as an avoidance mechanism, which might stabilize lipid bilayer under stress or ameliorate interaction between membranes. It has been proposed that freezing injury is caused by dehydration-induced apposition of chloroplast envelope to other organelle membranes, especially to the plasma membrane (Steponkus et al. 1993). In *P. patens* protonemata, the ABA-induced freezing tolerance was associated with increases in the cytoplasmic volume and reductions in lesions in plasma membrane and that an irreversible damage in the plasma membrane caused by freeze-induced dehydration was reduced in the ABA-treated protonema cells in comparison with the non-treated cells (Nagao et al. 2005). Increases in cytoplasmic volume were also observed in ABA-treated liverwort cells (Akter et al. 2014). Thus, reductions in the interaction of plasma membrane with membranes of other cellular organelles might be alleviated in the stress-tolerant bryophyte cells with an assistance of cytoplasmic components such as sugars and proteins (Nagao et al. 2005; Akter et al. 2014; Kadowaki et al. 2015).

10.7 Abscisic Acid

10.7.1 ABA as a Ubiquitous Stress Hormone in Land Plants

Various physiological studies on bryophytes have highlighted the pivotal role of abscisic acid (ABA) in stress responses in bryophytes. ABA is a sesquiterpene phytohormone that was identified as a compound that induces abscission of cotton leaves and also promotes dormancy of axillary buds of woody plants. ABA was shown to be involved in the regulation of a wide variety of developmental processes such as seed maturation and dormancy, stomata closure, and inhibition of the formation of lateral roots and inflorescence meristems (Milborrow 1974; Nambara and Marion-Poll 2010; Nambara et al. 2010). Above all, the role of ABA in the response to water deficit in vegetative plant tissues has attracted the attention of scientists investigating plant responses to environmental stresses such as drought, cold, and salinity (Rock et al. 2010). A decrease in soil water potential by drought accompanies an increase in endogenous levels of ABA in shoots, which causes closure of stomata to reduce transpiration (Wright and Hiron 1969; Cornish and Zeevaart 1986). In addition, exogenous ABA treatment increases the expression of a number of specific transcripts important for rendering stress tolerance to plants (Zeevaart and Creelman 1988).

Genetic and biochemical studies of tobacco, *Arabidopsis*, and maize have revealed the biosynthetic pathways for ABA in plants. ABA is synthesized from the C40 carotenoid precursor zeaxanthin (Fig. 10.1). The first step toward ABA biosynthesis is production of the epoxycarotenoids, antheraxanthin, and violaxanthin from zeaxanthin catalyzed by zeaxanthin epoxidase (ZEP). The epoxycarotenoids isomerized to 9-cis forms undergo oxidative cleavage by 9-cis-

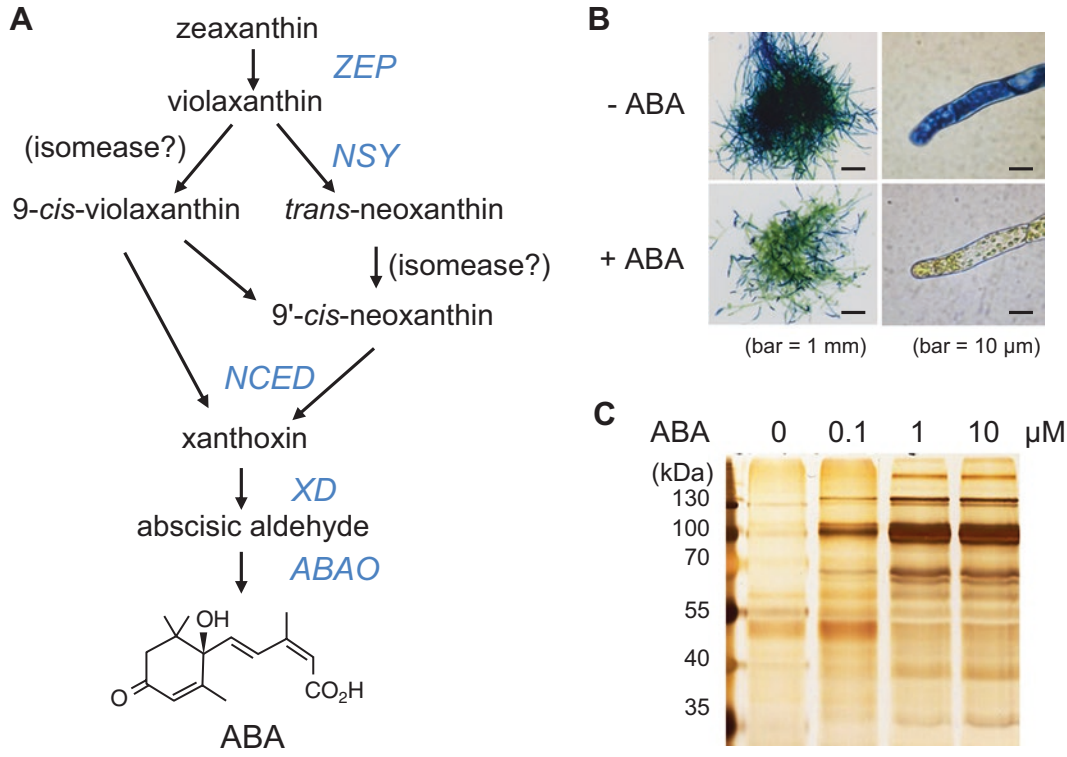

Fig. 10.1 Roles of ABA in desiccation tolerance and LEA-like protein accumulation in the moss *Physcomitrella patens*. (**A**) Biosynthetic pathway of ABA catalyzed by zeaxanthin epoxidase (ZEP), neoxanthin synthase (NSY), 9-*cis*-epoxycarotenoid dioxygenase (NCED), xanthoxin dehydrogenase (XD), and abscisic aldehyde oxidase (ABAO). (**B**) ABA (1 μM)-induced desiccation tolerance in *P. patens* protonemata analyzed by Evans blue staining. (**C**) Accumulation of LEA-like proteins by treatment of the *P. patens* protonemata with various concentrations of ABA

epoxycarotenoid dioxygenase (NCED) to give rise to a C15 compound xanthoxin. Xanthoxin is then oxidized to ABA by xanthoxin dehydrogenase (XD) and abscisic aldehyde oxidase (ABAO) (Seo and Koshiba 2002; Finkelstein 2013). Among these steps, cleavage of epoxycarotenoids by NCED is thought to be the rate-limiting step for synthesis of ABA (Nambara and Marion-Poll 2005). It has been shown that drought stress induces accumulation of *NCED* transcripts in various angiosperm species (Vishwakarma et al. 2017).

ABA is a compound that is found not only in land plants but also in various types of prokaryotic and eukaryotic organisms including cyanobacteria, eukaryotic algae, fungi, and lichens (Hartung 2010). However, high sensitivity responses and roles in water stress responses appear to be specific to land plants that comprise bryophytes and vascular plants (Takezawa et al. 2012). ABA has been detected in all three bryophyte groups, and its functions in stomata closure in sporophytes and enhancement of tolerance to desiccation or freezing in gametophytes have been demonstrated. Establishment of ABA as a "stress hormone" in bryophytes is likely to be achieved through evolutionary processes including acquisition of genes necessary for its synthesis and transport in early land plants (Takezawa et al. 2012; Sakata et al. 2014). High sensitivity responses to ABA also require molecules for perception, signal transduction, and ABA-regulated transcription of stress-associated genes in bryophytes.

10.7.2 Endogenous ABA in Bryophytes

Much effort has been devoted to measurement of endogenous ABA levels in bryophytes. By using monoclonal antibody-based assays, Hartung et al. (1987) found that a hornwort belonging to *Anthoceros* has endogenous ABA in both the gametophyte and sporophyte and that stress appears to induce ABA accumulation. They showed that stomata formed on the sporophyte of the hornwort were closed by exogenous ABA, suggesting a role of ABA in mediating water stress-induced closure of stomata. Hartung and Gimmler (1994) measured levels of ABA in several liverwort species and showed that ABA content in liverworts varies greatly among species. They estimated that thalloid liverwort species accumulate endogenous ABA with a content ranging from 1 to 10 pmol/gFW in the semi-aquatic liverwort *Riccia fluitans* to 30 nmol/gFW in the xerophytic liverwort *Exormotheca*. Although immunological methods are suitable for surveys of ABA in many species, more reliable analysis by gas chromatography-mass spectroscopy (GC-MS) of cultured bryophytes free from other organisms enabled researchers to precisely measure the amount of endogenous ABA in bryophytes. Estimation by GC-MS of endogenous ABA content in the liverwort *M. polymorpha* by Li et al. (1994) showed that the liverwort contains 4–16 ng gFW^{-1} (15–60 pmol gFW^{-1}) of ABA. These values are consistent with our recent analysis by GC-MS indicating that cultured thalli of *M. polymorpha* contain 6.5–9.9 ng gFW^{-1} (24–37 pmol gFW^{-1}) of ABA (unpublished results).

For mosses, ABA levels in protonemata of *Funaria hygrometrica* were estimated by Werner et al. (1991) using ELISA. The ABA level was increased from 1.7 to 10.5 nmol gFW^{-1} after 20 h of slow desiccation. ABA measurement by GC-MS was carried out by Minami et al. (2005) using cultured protonemata of *P. patens*, and it was shown that the protonemata had 2.4 ng gDW^{-1} of ABA and that the content was increased to 5.1 ng gDW^{-1} by treatment with the hyperosmotic mannitol, which increased freezing tolerance of the protonemata. The pathway for ABA biosynthesis might be similar to that of angiosperms, because the *ppaba1* mutant of *P. patens* lacking zeaxanthin epoxidase, which catalyzes the initial reaction toward ABA biosynthesis (Fig. 10.1A), was not capable of accumulating detectable amounts of endogenous ABA (Takezawa et al. 2015). Drábková et al. (2015) reported the results of evaluation of phytohormones in 30 bryophyte species. Their results showed that ABA was present in all of the bryophyte samples tested at concentrations ranging from 1.02 pmol g FW^{-1} in the moss *Sphagnum compactum* to 302.22 pmol g FW^{-1} in the moss *Calliergonella cuspidata*.

10.7.3 ABA-Induced Physiological Responses

Werner et al. (1991) demonstrated a role of ABA in desiccation tolerance of *F. hygrometrica*, for which cultured protonemata are sensitive to rapid desiccation but tolerant to slow desiccation. By treatment with ABA, the protonemata acquired tolerance to rapid desiccation, indicating that ABA is involved in inducible desiccation tolerance that develops along with slow desiccation. ABA also induced desiccation tolerance in the moss *Atrichum androgynum* (Beckett 1999), to the same degree as that induced by partial dehydration. This indicated that ABA is involved in the inducible mechanisms for desiccation tolerance. In *A. undulatum*, ABA enhanced the tolerance of photosystem II to desiccation and increased non-photochemical quenching upon rehydration (Beckett et al. 2000; Mayaba et al. 2001). We reported that ABA treatment of *P. patens* induces freezing tolerance of protonema tissues (Minami et al. 2003a, b). ABA also induces desiccation tolerance with accumulation of various types of LEA-like proteins (Fig. 10.1). Accumulation of various transcripts including those of *LEA*-like genes and those with similarity to stress-associated genes of angiosperms was induced by ABA treatment in the *P. patens* protonema cells (Minami et al. 2003a, b; Cuming et al. 2007). The protonema cells were damaged by

exposure to air with RH below 91% (−13 MPa) due to membrane damage but withstood 13% RH (−273 MPa) when treated with ABA (Koster et al. 2010).

The role of ABA in desiccation tolerance in liverworts was reported by Hellwege et al. (1994), who showed that exogenous ABA treatment of the xerophytic liverwort *Exormotheca holstii* resulted in rapid recovery of photosynthesis after desiccation and rehydration. Reduction in ABA levels upon hydration was also obvious. Effects of ABA pretreatment on survival after cryopreservation and desiccation tolerance in different liverwort species (Pence 1998, 2005) in association with sugar accumulation have already been mentioned. It appears that there are differential effects of ABA on liverwort species and tissues (Pence et al. 2005; Akter et al. 2014).

10.7.4 Molecular Mechanisms Underlying ABA and Stress Responses in Bryophytes

As mentioned above, ABA treatment in bryophytes causes enhancement of tolerance to desiccation and freezing, and thus recovery from cryopreservation, in association with accumulation of soluble sugars and transcripts encoding LEA-like proteins and other stress-associated proteins in both mosses and liverworts. These physiological changes indicate that these bryophytes possess molecules for perception, signal transduction, and transcriptional activation in response to ABA. In *Arabidopsis*, gene expression induced by ABA is mediated by a specific *cis*-promoter element called ABRE (for ABA-responsive element) with an ACGT core motif recognized by transcription factors AREB (for ABA-responsive element binding) factors (Fujita et al. 2011). Molecular aspects of ABA responses in bryophytes were extensively studied in the model plant *P. patens* (Cove 2005). ABA responses of protonemata of *P. patens* were analyzed by Knight et al. (1995), and they showed that the protonemata respond to ABA and osmotic stress to activate the ABA-inducible *Em* promoter fused to the beta-glucuronidase (*GUS*) reporter gene. The presence of a transcription factor that binds to ABRE in the promoter was also suggested. Cuming et al. (2007) carried out microarray analysis of the ABA-induced transcriptome of *P. patens* and showed that an ABRE-like sequence in the promoter was overrepresented in many ABA-induced genes. Elimination of the ACGT core motif from ABRE resulted in a drastic reduction of the ABA response (Sakata et al. 2010). Overlapping expression profiles, as well as upregulation of ABA biosynthesis genes, suggest that ABA mediates the salt stress responses in *P. patens*. A global expression study by microarray and RNA-seq analyses also indicated overlaps of transcripts induced by ABA with cold, salinity, and drought stress (Richardt et al. 2010; Khraiwesh et al. 2015). An important role of ABRE in liverworts has also been suggested. Analysis of the promoter of *M. polymorpha DHN* gene (*MpDHN1*) revealed that the promoter possesses multiple ABRE-like motifs, deletion of which resulted in reduction in ABA-induced gene expression (Ghosh et al. 2016a, b).

It has been shown that ABA provokes stress responses through binding to the intracellular receptors PYR/PYL/RCARs. Mutants of PYR/PYL/RCARs exhibit reduced sensitivity to ABA in seed germination and stomatal closure (Ma et al. 2009; Park et al. 2009; Gonzalez-Guzman et al. 2012). The ABA-receptor complex inhibits group A protein phosphatase 2C (PP2C), a negative regulator of ABA signaling (Gosti et al. 1999), and inhibition of PP2C causes activation of subclass III SnRK2, which is a central regulatory kinase that phosphorylates and activates various key cellular molecules including AREB for ABA-induced gene expression (Cutler et al. 2010; Umezawa et al. 2011).

Phylogenetic analysis of both *P. patens* and *M. polymorpha* indicated that the genes for the core signaling molecules for the initial ABA response are likely to be conserved in bryophytes (Sakata et al. 2014; Bowman et al. 2017). *P. patens* and *M. polymorpha* genomes have four and five *PYR/PYL/RCAR*-like genes, respectively. We showed that one of the genes for the *M. polymorpha PYR/PYL/RCAR*-like gene (Mp*PYL1*) fused to *GFP* (Mp*PYL1-GFP*) complemented an ABA-

insensitive phenotype of the *pyr1pyl1pyl2pyl4* quadruple mutant of *Arabidopsis* (Bowman et al. 2017). Overexpression of Mp*PYL1* in Marchantia callus cells resulted in activation of an ABA-inducible promoter via ABRE in transient gene expression assays. Furthermore, a transgenic *M. polymorpha* line overexpressing Mp*PYL1-GFP* was hypersensitive to ABA and more tolerant than the wild type to desiccation, while a line lacking Mp*PYL1* (Mp*pyl1*) was sensitive to desiccation even after ABA treatment. Furthermore, of 35 ABA-induced *LEA*-like transcripts, expression of 32 transcripts was increased in the overexpression lines and the expression of all 35 transcripts was decreased in the Mp*pyl1* line (Jahan et al. unpublished results). A role of reversible phosphorylation by PP2C and SnRK2 in ABA signaling is also likely to be conserved in bryophytes. Overexpression of group A PP2C resulted in loss of tolerance to salt and hyperosmotic stress and insensitivity to ABA in transgenic *P. patens* (Tougane et al. 2010) and reduced accumulation of LEA-like proteins in transgenic *M. polymorpha* (Kadowaki et al. 2015). Disruption of the genes for the group A PP2C resulted in constitutive desiccation tolerance in *P. patens* (Komatsu et al. 2013). We recently isolated the "AR7" mutant of *P. patens* with little SnRK2 activity. The AR7 cells were less tolerant to freezing and hyperosmotic stress with reduced ABA sensitivity (Minami et al. 2006; Saruhashi et al. 2015). AR7 has a mutation in the *ARK* (for ABA and abiotic stress response kinase) gene encoding a Raf-like protein kinase (Saruhashi et al. 2015). Bacterially expressed proteins of the ARK kinase domain phosphorylated and activated one of the SnRK2 isoforms, PpSnRK2B, indicating that ARK is an upstream regulator of SnRK2 (Fig. 10.2). Gene expression studies indicated that not only responses to ABA but also responses to hyperosmosis were impaired in the mutant deficient in ARK, indicating that ARK mediates the both signaling processes.

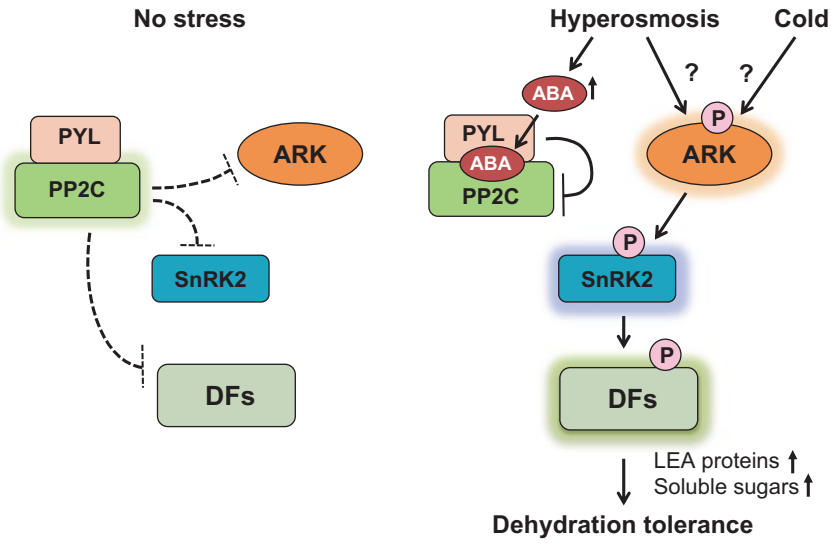

Fig. 10.2 Hypothetical model representing the phosphorylation-mediated control of dehydration tolerance in bryophytes. ARK, SnRK2, and downstream factors (DFs) are activated by phosphorylation, which is triggered by hyperosmosis, cold, and endogenous ABA, for development of dehydration tolerance. Without stress, phosphorylation of above factors is inhibited by PP2C. It has been shown that ABA inhibits PP2C via binding to the PYR/PYL/RCAR receptor (PYL), but how hyperosmosis and cold activate phosphorylation has not been clarified

10.8 Responses to Low Temperature

10.8.1 Cold Acclimation in Bryophytes

Biennial as well as perennial plant species in the temperate and sub-frigid zones acclimate to cold temperatures and develop freezing tolerance over late autumn and early winter. Capacity for cold acclimation can be estimated in the laboratory by exposing plants to temperatures of 2–4 °C for several days to weeks and determining changes in freezing tolerance (Levitt 1980; Sakai and Larcher 1987). Cold acclimation provokes various biochemical and physiological changes in plant cells, including expression of stress-associated transcripts, typically those of cold-responsive (*COR*) genes, accumulation of compatible solutes and antioxidants accompanied by changes in membrane lipid composition, reduction of tissue water content, activity of various metabolic enzymes, and "augmentation" of intracellular structures (Pearce 1999). These changes are brought about by sensing of low temperatures and provoking molecular mechanism for regulation of gene expression through cold-specific signaling processes. Endogenous ABA might play a role in mediating cold acclimation, since there are several reports showing that ABA levels increase during cold acclimation in various plant species (Daie and Campbell 1981; Lalk and Dörffling 1985).

Bryophytes also appear to undergo cold acclimation. Dircksen (1964) showed that bryophyte species exhibit freezing tolerance 9–10 °C greater in winter than in summer. Rütten and Santarius (1992a, 1993) examined freezing tolerance of seven moss species and one liverwort species in summer and winter. While the freezing tolerance of the mosses differed in summer and winter by 15 °C to more than 25 °C, a seasonal difference in the liverwort was relatively small. These studies indicate that bryophytes in a natural habitat can sense low temperature and provoke specific responses to enhance freezing tolerance, although the naturally acclimated samples are affected by other environmental signals such as humidity and light. Laboratory experiments were conducted using an axenic protonemal culture of *P. patens*. When the protonemata of *P. patens* were exposed to different low temperatures (15 °C, 10 °C, 4 °C and 0 °C) under continuous light, acclimation at 0 °C increased freezing tolerance greater than other temperature treatments and that 15 °C did not increase freezing tolerance. The *P. patens* gametophore also acclimates to cold, and the levels of freezing tolerance achieved by cold acclimation in the gametophore were higher than the tolerance levels in the protonemata (Sun et al. 2007). The increase in freezing tolerance during cold acclimation accompanied an accumulation of various *LEA*-like transcripts, which are also inducible by ABA and hyperosmosis (Minami et al. 2005), indicating that cold and hyperosmosis signals share the same pathway for the gene expression. However, more comprehensive analysis revealed that there might be a pattern of cold-specific regulation of transcripts that is distinct from that of ABA or other abiotic stress responses. Comparative proteomic analysis showed that the response to cold was quite different from the responses to drought and salinity (Wang et al. 2009a, b). Transcriptome analysis also indicated that there are distinct transcripts specifically induced by cold, some of which are not commonly induced by cold in angiosperms. It was found that 12% of cold-responsive genes in *P. patens* had no orthologs in other plants (Beike et al. 2015).

10.8.2 Molecular Response to Cold

Mechanisms underlying cold-responsive gene expression have been studied in angiosperms, especially in *Arabidopsis*. Expression of *COR* genes encoding LEA-like proteins is driven by the transcription factor C-repeat binding factor (CBF), also known as a dehydration-responsive element binding (DREB) transcription factor, with a conserved AP2/ERF DNA-binding domain (Stockinger et al. 1997). Overexpression of the stress-responsive transcription factors CBF1/DREB1B and CBF3/DREB1A in transgenic *Arabidopsis* plants resulted in constitutive

expression of COR genes and increased tolerance to freezing and drought (Jaglo-Ottosen et al. 1998; Kasuga et al. 1999). Ectopic expression of these transcription factors can enhance freezing tolerance in other angiosperm plants such as tobacco (Kasuga et al. 2004), barrel clover (Chen et al. 2010), and maize (Zhang et al. 2010), indicating that the role of CBF/DREB in cold responses is common in angiosperms. *P. patens* possesses a gene for the AP2/ERF transcription factor PpDBF1, and overexpression of *PpDBF1* in transgenic tobacco plants resulted in higher tolerance to salt, drought, and cold stresses than the wild type (Liu et al. 2007). DREB-related genes were also identified in the desert moss *Syntrichia caninervis*, and it was shown that these genes conferred salt, cold, osmotic, and heat tolerance when expressed in yeast cells (Li et al. 2016). However, there is no direct evidence showing that CBF/DREB-type transcription factors activate cold-induced genes in mosses.

10.8.3 Role of ABA in Cold Acclimation in Bryophytes

Whether ABA mediates cold responses in plants has been an issue of controversy (Gusta et al. 2005). In *P. patens* protonemata, many cold-induced genes contain a putative ABRE in promoters (Cuming et al. 2007), indicating that ABA might mediate cold signals. However, an apparent increase in endogenous ABA levels was not observed during 7-day cold acclimation in protonemata (Minami et al. 2005). The level of freezing tolerance after cold treatment for 7 days (LT50 = −3.5 °C) was much lower than that achieved by 1-day ABA treatment (LT_{50} = −8 °C) (Minami et al. 2003a). Analysis of an ABA-less *ppaba1* mutant of *P. patens* indicated that while cold-induced accumulation of transcripts for three *LEA*-like genes was reduced in the mutant, freezing tolerance capacity in the mutant protonemata was similar to that in the wild type (Takezawa et al. 2015). It was found that *ppaba1* protonemata accumulate soluble sugars to levels similar to those in the wild type during cold acclimation. These findings indicate that soluble sugars that accumulated during cold acclimation are crucial for freezing tolerance, and this process does not require endogenous ABA. In the gametophore, a fourfold increase in the level of ABA was observed after 2 days of cold acclimation (Beike et al. 2015), indicating that ABA might contribute to the enhancement of freezing tolerance during cold acclimation. Changes in freezing tolerance or sugar levels in the cold-acclimated gametophore of *ppaba1* have not been examined.

Although the role of endogenous ABA in cold acclimation of mosses is not yet conclusive, a signal crosstalk between ABA and cold is likely to be present in *P. patens*. Bhyan et al. (2012) analyzed cold acclimation capacity of an AR7 mutant with little SnRK2 activity (Minami et al. 2006) and a D2-1 line overexpressing the catalytic domain of Group A PP2C (Tougane et al. 2010). Both lines had less capacity of cold acclimation than that of the wild type with reduced accumulation of stress-associated transcripts and soluble sugars (Bhyan et al. 2012). These results indicate that cold and ABA responses share common signaling pathways, possibly regulated by a reversible protein phosphorylation (Fig. 10.2). Reduced acclimation capacity of AR7 with a mutation in ARK indicated that activation of SnRK2 by ARK is crucial to cold acclimation in bryophytes. Our recent analysis indicated that ARK expression is increased by cold (unpublished results). However, the way cold regulates activity of ARK and downstream molecules leading to induction of gene expression and sugar accumulation has not been clarified. We also found that some Raf-like kinases of *A. thaliana* and the lycophyte *Selaginella moellendorffii* have functions similar to ARK, implicating a conserved role of the kinase in ABA as well as cold signaling in vascular and nonvascular plants (Saruhashi et al. 2015).

10.9 Concluding Remarks

A series of ecological and physiological studies on bryophytes with various levels of tolerance to desiccation and freezing revealed a landscape of their survival strategy to dehydration that is

distinct from that of vascular plants. Resemblance with resurrection vascular plants of photosynthetic responses and cytological characteristics indicates cellular mechanisms common in plant cells with high desiccation tolerance. Further studies for clarification of the core mechanism underlying desiccation tolerance at the cellular level are necessary to answer a critical and yet unanswered question: why are bryophytes so tolerant to dehydration? In this respect, bryophytes with simple structures are ideal for molecular cytological studies of desiccation tolerance (Pressel and Duckett 2010), in combination with techniques of molecular labeling and genetic manipulation. About responses to low temperatures, tolerance to freezing in bryophytes depends on the levels of hydration, and interpretation of experimental data made in the laboratory requires a consideration of ecological behavior of each species. Nevertheless, it is obvious that bryophytes undergo cold acclimation with accumulation of specific transcripts and low-molecular-weight soluble sugars, similar to vascular plants. This indicates that land plants at least share some evolutionarily conserved mechanisms for cold responses, although details of the mechanisms including those for sensing temperature drops and activating cold-responsive genes have not been clarified. Molecular physiological studies on the model bryophytes *P. patens* and *M. polymorpha* have highlighted a functional diversification of the conserved molecules in land plants (Komatsu et al. 2013; McAdam et al. 2016). Technical advances in genomic, cytological, and biochemical analyses now provide us with an opportunity to take a multi-angle approach to clarify cellular mechanisms for high stress tolerance in bryophytes and further improve our knowledge on the growth and survival strategies of land plants.

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11

Regulatory Gene Networks in Drought Stress Responses and Resistance in Plants

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Abstract

Plant responses to drought stress have been analyzed extensively to reveal complex regulatory gene networks, including the detection of water deficit signals, as well as the physiological, cellular, and molecular responses. Plants recognize water deficit conditions at their roots and transmit this signal to their shoots to synthesize abscisic acid (ABA) in their leaves. ABA is a key phytohormone that regulates physiological and molecular responses to drought stress, such as stomatal closure, gene expression, and the accumulation of osmoprotectants and stress proteins. ABA transporters function as the first step for propagating synthesized ABA. To prevent water loss, ABA influx in guard cells is detected by several protein kinases, such as SnRK2s and MAPKs that regulate stomatal closure. ABA mediates a wide variety of gene expression machineries with stress-responsive transcription factors, including DREBs and AREBs, to acquire drought stress resistance in whole tissues. In this chapter, we summarize recent advances in drought stress signaling, focusing on gene networks in cellular and intercellular stress responses and drought resistance.

Keywords

Dehydration · Abscisic acid (ABA) · Root-to-shoot signaling · Stomatal closure · Gene expression

Abbreviations

ABA	Abscisic acid
ABCG	ATP-binding cassette G
AREB	ABRE-binding protein
CBLs	Calcineurin B-like proteins
CDPKs/CPKs	Ca^{2+}-dependent protein kinases
CIPKs	CBL-interacting protein kinases
DREB	DRE-binding protein
HK	Histidine kinase
MAPKs/MPKs	Mitogen-activated protein kinase
PYL	PYR1-like
PYR	Pyrabactin resistance
RCAR	Regulatory component of ABA receptors
SnRK2	SNF1-related protein kinase 2
WUE	Water use efficiency

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11.1 Introduction

Plants are sessile organisms that require them to adapt to severe environmental conditions for development and survival. For higher plants to achieve optimal growth maintenance, various mobile molecules are required to propagate extracellular stimuli from the detecting tissue to the target. In land plants, local and long-distance signaling via small molecules is important to prevent water loss by transpiration from guard cells and to adapt to drought stress conditions (Steudle 2001; Christmann et al. 2013).

Abscisic acid (ABA) is a key phytohormone responsible for drought stress resistance by regulating stomatal movement and gene expression, leading to cellular adaptation to water deficit conditions. ABA has been discovered as a growth-inhibiting plant hormone in the 1960s (Addicott et al. 1968). In addition to its role in the drought response, ABA is an essential hormone throughout the entire lifespan of a plant, including seed dormancy, germination, post-germination growth, and maturation (Cutler et al. 2010). ABA accumulates under drought stress conditions and quickly spreads among intercellular spaces in tissues. Plasma membrane-type ABC transporters have been identified and mediate the influx/efflux system of ABA (Kang et al. 2010; Kuromori et al. 2010; Kuromori and Shinozaki 2010). Therefore, ABA has been suggested as a candidate mobile molecule that transmits drought stress information into plant cells.

Recent studies have revealed core components of ABA signaling. A pyrabactin resistance 1 (PYR)/PYR1-like (PYL)/regulatory component of ABA receptors (RCAR) has been identified as an intracellular ABA receptor (Ma et al. 2009; Park et al. 2009; Santiago et al. 2009). PYR/PYL/RCAR belongs to the steroidogenic acute regulatory protein-related lipid transfer (START) domain protein family. There are 14 homologous genes of PYR/PYL/RCAR in the *Arabidopsis* genome (McConnell et al. 2001). In addition to PYR/PYL/RCAR, protein phosphatase 2Cs (PP2Cs) and sucrose nonfermenting 1 (SNF1)-related protein kinase 2 (SnRK2) also act to perceive ABA. These three components coordinate ABA signal transduction by regulating SnRK2s activity to achieve cellular adaptation in response to dehydration stress. Active SnRK2s phosphorylate various substrates that comprise a major protein phosphorylation network in ABA signaling.

ABA accumulation in response to dehydration stress influences expression of several genes with various functions in stress tolerance. In these gene expression processes, two types of transcription factors, ABRE-binding proteins (AREBs)/ABRE-binding factors (ABFs) and DRE-binding proteins (DREBs), regulate ABA-inducible and/or dehydration stress-inducible gene expression. AREBs/ABFs and DREBs require posttranslational modification for their activation in response to ABA and/or dehydration stress (Uno et al. 2000; Qin et al. 2008). Several studies have shown that SnRK2s phosphorylate and activate AREBs/ABFs in ABA signaling in response to water deficit signals (Furihata et al. 2006; Fujii et al. 2007). It has been proposed that SnRK2s-AREBs/ABFs constitute the main signal transduction pathway in ABA signaling.

In this chapter, we review recent advances in drought stress signaling, focusing primarily on ABA transport, ABA signal transduction via phosphorylation of protein kinases, and transcription factor-regulated gene expression networks in drought stress responses.

11.2 Intercellular Regulatory Networks in Drought Stress Responses and Resistance

11.2.1 Long-Distance Signaling in Drought Stress

11.2.1.1 ABA Signaling in Long-Distance Communication

Plants are sessile and should have evolved the capacity to sense and respond to various stresses of changing environments. It is very important to understand how intercellular regulation is

achieved in plants, because intercellular signals function above the cellular level and affect distant tissues and the whole plant (Busch and Benfey 2010). Specifically, plants absorb water through their roots. The water leaves the plant through its leaves via transpiration in the shoot. Most higher plants transpire through their stomata located in epidermal tissues. Thus, stomatal movements can be controlled by remote signaling in plants. Indeed, it has been documented that stomatal behavior is remotely regulated in response to a variety of environmental stresses, including root-to-shoot signaling under drought stress (Jia and Zhang 2008).

In early experiments, stomatal closure occurred when a part of the root system was exposed to water deficits by a root-split experiment, even though the water status in the leaves remained unchanged (Wilkinson and Davies 2002). These findings indicate that a root-derived signal was transported to the leaves, inducing stomatal closure. While the substance responsible for root-to-shoot signaling under drought stress has not completely been identified, several candidate substances exist and are described below.

ABA is one of the most promising candidates as the root-to-shoot signaling molecule. Under mildly stressful conditions (e.g., when soil drying begins), ABA accumulates in root tissues, and increased ABA levels are correlated with a decrease in leaf stomatal conductance (Taiz and Zeiger 2010). In addition, drought stress induces a drastic increase in ABA content in the xylem sap (Zhang and Davies 1990; Jiang and Hartung 2008). These previous results provide a working hypothesis: ABA is released to the xylem vessels and transported in the xylem as a long-distance signal through the xylem stream to remotely regulate stomatal movement under drought stress.

ABA is a weak acid that is ionized in alkaline section and compartmentally redistributed by a pH gradient across lipid membranes. Provided that pH changes can be induced by drought stress, pH changes may act as a signal to regulate stomatal movement. Indeed, the pH of the xylem sap has been shown to increase in response to dry soil conditions in many plant species (Wilkinson 1999; Jia and Davies 2007). These data suggest that pH changes may signal coordinated changes with the ABA signal under drought stress that regulates stomatal movement, while stomatal regulation by pH signaling has not been much described yet with the exception of drought stress (Jia and Zhang 2008).

11.2.1.2 Other Chemical Candidates or Physical Transmitters Related to Root-to-Shoot Communication

Besides ABA, a variety of substances have been proposed as chemical alternatives for xylem-borne stress signals (Fig. 11.1). ABA conjugates with glucose to form ABA glucosyl ester (ABA-GE), which has been detected in the xylem sap of various plants, and may serve as another transportable candidate to function in root-to-shoot signaling under stress (Sauter et al. 2002; Xu et al. 2013a; Dong et al. 2015). Because of its extremely hydrophilic properties, ABA-GE can be transported over long distances through the stem xylem, without loss to the surrounding parenchyma (Jiang and Hartung 2008). On the other hand, while membrane transporters responsible for ABA-GE transport have been described, it has been argued that the amount of ABA-GE in roots is too small to contribute significantly to the overall increase in ABA during water stress (Goodger and Schachtman 2010; Burla et al. 2013).

Cytokinin is another phytohormone that is known to be synthesized mainly in roots and has antagonistic effects to ABA. Some reports suggest that cytokinin is correlated with stomatal control in particular plants (Vysotskaya et al. 2004; Davies et al. 2005). Acetylcholine is another candidate for the long-distance signal, because it is a nerve transmitter in animals that has physiological functions in plants (Tretyn and Kendrick 1991; Madhavan et al. 1995). Both cytokinin and acetylcholine have been suggested to play roles in the regulation of stomatal movement. However, their role in the drought response remains unclear. Malate is another potential candidate: It is a xylem sap constituent known to increase in response to water stress and is involved in the guard cell signal transduction

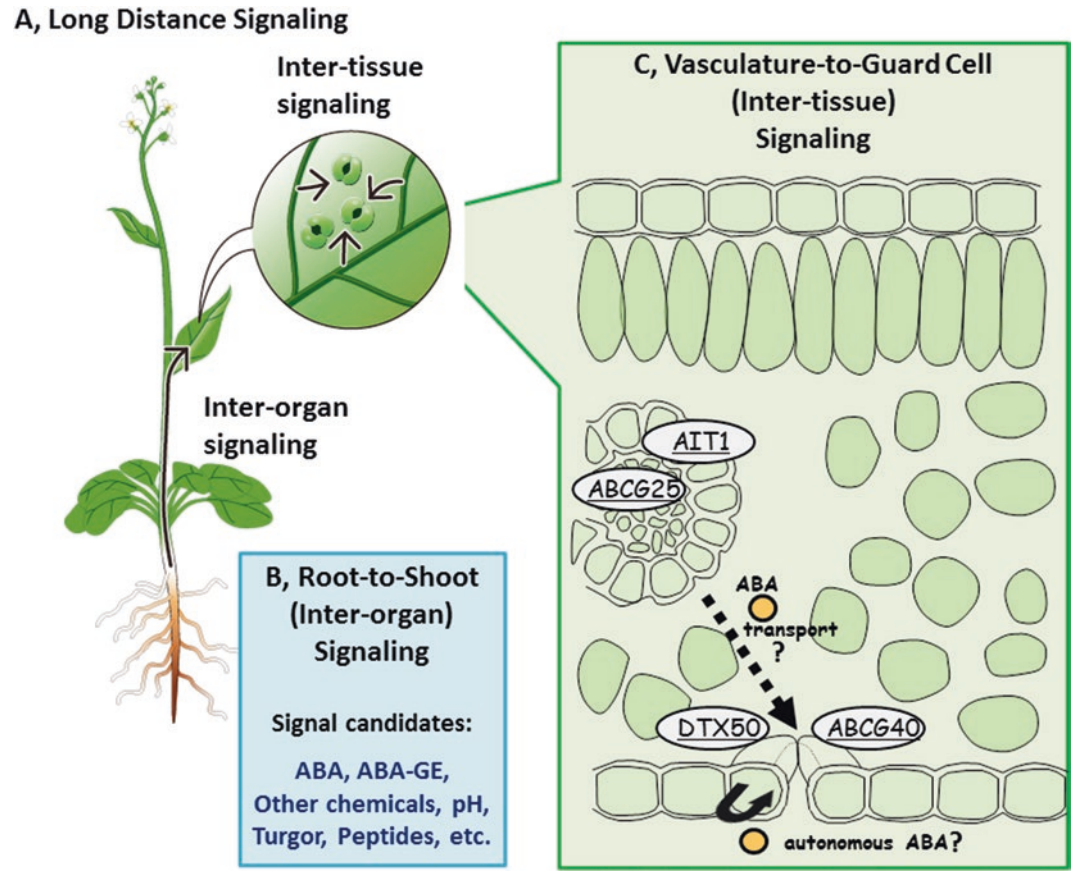

Fig. 11.1 Systemic signaling networks in plant drought stress responses. (**a**) Long-distance signaling is composed of regional divisions that include inter-organ signaling and inter-tissue signaling. (**b**) Root-to-shoot (inter-organ) signaling has been discussed as a form of systemic signaling in the plant drought responses. Likely, it is composed of several substances, including ABA and other chemical or physical regulators. (**c**) Vasculature-to-guard cell (inter-tissue) signaling is considered as an ABA regional network, especially in leaves, because several ABA transporters (such as ABCG25, ABCG40, AIT1, and DTX50) were expressed in vascular cells and/or guard cells. On the other hand, the effect of autonomous ABA in the guard cells is also a possibility

network (Hedrich and Marten 1993; Hedrich et al. 1994; Patonnier et al. 1999; Kim et al. 2010). Nevertheless, the xylem sap malate concentration increases significantly only in the later stages of water stress (Goodger and Schachtman 2010). In studies of xylem sap constituents, sulfate, but not ABA, has been observed to increase as a result of early water stress (Goodger and Schachtman 2010; Malcheska et al. 2017). Thus, further investigations are necessary to provide insight into the first signals responsible for drought stress sensing.

Chemical signals have been demonstrated to be responsible for root-to-shoot signaling in response to soil drying. However, there are some evidences supporting a water-related physical signal, such as a hydraulic signal, is a proposed substance responsible for root-to-shoot signaling (Jiang and Hartung 2008). In early experiments, soil drying-induced reductions in leaf conductance could be progressively reversed by the pressurization of the root system (Fuchs and Livingston 1996). A more recent experiment in *Arabidopsis* has shown that stomatal closure

induced by root-applied water deficits could be relieved by supplying water directly to the leaves (Christmann et al. 2007). This indicates that hydraulic signals can serve as a regulatory component that affects stomatal behavior, which better fits the model for woody plants, which have long distances between the root and shoot (Saliendra et al. 1995).

Recently, we identified one of the hydrophilic peptides that mediates ABA accumulation in leaves in response to drought stress (unpublished data). The peptide is expressed mainly in vascular tissues of roots. The root-derived peptide is recognized by several receptor-like kinases in the leaves, and it regulates gene expression of *9-cis-epoxycarotenoid dioxygenase 3* (*NCED3*), which is a key enzyme of ABA biosynthesis in leaves. This peptide has been proposed as additional candidate for the root-to-shoot signaling in response to drought stress conditions.

11.2.2 Abscisic Acid Transport in Inter-tissue Signal Transfer

11.2.2.1 ABA Biosynthesis and Signaling in Leaves

While the substance responsible for root-to-shoot signaling under drought stress remains under debate, ABA is one of the known factors that promotes stomatal closure in guard cells to prevent transpiration under drought conditions (Leung and Giraudat 1998; Cutler et al. 2010; Kim et al. 2010). The explanation found in many textbooks is that ABA is produced mainly in the roots upon water limitation, loaded into the xylem vessels, and transported shootward by the transpiration stream, where it acts on guard cells to induce stomatal closure (Taiz and Zeiger 2010). However, recent data have added new levels of complexity to this widely accepted hypothesis (Lacombe and Achard 2016). For example, three pieces of experimental data argue that leaves may actually be the principal sites of ABA biosynthesis. First, it has been suggested that ABA biosynthesis enzymes are expressed at high levels in leaves (Iuchi et al. 2001; Endo et al. 2008a, b). Moreover, additional studies support leaves as the main site of ABA biosynthesis, whereas ABA production in roots appears to be rather limited (McAdam et al. 2016a, b; Sussmilch et al. 2017). Second, reciprocal grafting between ABA-deficient mutants and wild-type plants demonstrated that stomatal closure was affected by the leaf genotype, but not the root genotype, arguing that ABA biosynthesis in the shoot was necessary and sufficient to mediate stomatal closure of plants that were water stressed at the roots (Holbrook et al. 2002; Christmann et al. 2007). Third, as demonstrated in several species, ABA content in the roots under long-term or continued periods of water stress relies largely on basipetal transport of ABA from aerial organs, as ABA accumulation exclusively in roots depends on aboveground tissues (Ren et al. 2007; Ikegami et al. 2009; Manzi et al. 2015, 2016). Recently, these observations have been confirmed using an optogenetic technique showing that the ABA-specific reporter allows direct cellular monitoring of dynamic ABA concentration changes in response to environmental stresses (Jones et al. 2014; Waadt et al. 2014).

In addition to the observation that leaves are the primary organ of ABA biosynthesis, more tissue-specific analyses suggest that ABA biosynthesis enzymes are expressed in the vascular bundles of leaves (Endo et al. 2008a, b; Kuromori et al. 2014). Under water shortage, ABA responses are finally induced throughout the leaves, including the epidermal guard cells, which suggests that ABA signaling may be distributed across leaves. In this case, ABA may be transmitted from the site of biosynthesis to the site of action in leaves as a form of middle-distance communication, rather than for long-distance communication in root-to-shoot signaling.

11.2.2.2 ABA Transporters

Several ABA membrane transporters have been reported to date at the molecular level (Boursiac et al. 2013). Based on mutant phenotyping

approaches, two ABA transporters, *AtABCG25* and *AtABCG40*, which belong to the ATP-binding cassette transporter family, have been characterized biochemically. These analyses have revealed that *AtABCG25* mediates ABA export from the cell and *AtABCG40* mediates import into the cell (Kang et al. 2010; Kuromori et al. 2010; Kuromori and Shinozaki 2010). The promoter of *AtABCG25* is active in the vascular tissue, and the *AtABCG40* promoter is active in guard cells. This is consistent with the hypothesis that the primary site of ABA synthesis is vascular tissues that is then transported to guard cells. However, mutants defective in each of these transporters do not perfectly match typical phenotypes of ABA-deficient mutants. This suggests the presence of redundant transporters or passive transport mechanisms mediated by pH gradients. Indeed, in mature seeds, *AtABCG31* and *AtABCG30*, as well as *AtABCG25* and *AtABCG40*, have been shown to serve as supplementary ABA exporters and importers, respectively (Kang et al. 2015).

In a different family of membrane transporters, AIT1 and members of the nitrate transporter 1/peptide transporter (NRT1/PTR) family have been identified as additional ABA importers (Kanno et al. 2012; Chiba et al. 2015; Tal et al. 2016). In addition, DTX50, which belongs to the multidrug and toxin efflux transporter (MATE) family, has been identified to be an additional ABA exporter (Zhang et al. 2014). These findings strongly suggest an active control of ABA transportation by plasma membrane carriers. These multiple ABA transporters, including both exporters and importers, might manage the ABA intercellular signaling in the bodies of plants. This model is consistent with findings that cellular ABA receptors that trigger ABA signaling are soluble and localized to the cytosol (Ma et al. 2009; Park et al. 2009).

In contrast, another report proposed that guard cells in the leaf epidermis are directly autonomous for ABA synthesis in response to changes in leaf hydration (Bauer et al. 2013). Guard cells are probably sensitive to changes in aerial humidity (Merilo et al. 2015). At this point, in addition to not knowing exactly where water status is sensed in the leaves, the routes of ABA transport have not been elucidated completely, providing an impetus for future studies that examine ABA intercellular regulation.

11.2.3 Applications to Improve Water Use Efficiency (WUE)

To date, there have been several attempts to improve stress tolerance, particularly drought tolerance, by overexpression of stress-related genes in transgenic plants (Umezawa et al. 2006). However, a common obstacle in this approach has been that the key factors have strongly negative effects on intrinsic plant growth. For example, the overexpression of an ABA biosynthesis enzyme or a transcription factor that controls many genes under abiotic stress produced dwarfed or growth-retarded phenotypes under unstressed conditions (Kasuga et al. 1999; Iuchi et al. 2001). A strategy that overcomes this issue would be useful for breeding drought-tolerant plants.

Recent reports have demonstrated that regulation of intercellular ABA transport or inter-tissue signaling may be useful for generating novel breeding technologies to improve both drought tolerance and water use efficiency of plants without growth retardation (Kuromori et al. 2016). When the cell-membrane ABA transporter *AtABCG25* was overexpressed in plants, the transgenic plants showed a reduced-transpiration phenotype with enhanced drought tolerance. This was probably a result of better maintenance of water contents after drought stress. Interestingly, *AtABCG25*-overexpressing plants did not appear to show growth retardation, unlike many previously reported transgenic plants that overexpressed genes involved in drought stress. Finally, it has been shown that this unique trait of the *AtABCG25*-overexpressing plants resulted from enhanced water use efficiency (WUE), i.e., greater biomass production per amount of water used (Yoo et al. 2010). This indicates that *AtABCG25* is a positive regulator of WUE.

In addition to improving drought tolerance, the improvement of water use efficiency is a major challenge in plant physiology research.

Due to their trade-off relationship, it is generally considered that achieving stress tolerance is incompatible with maintaining stable growth. *AtABCG25*-overexpressing plants showed a lower transpiration phenotype without any growth retardation. This may be a useful example for improving the water use efficiency and drought tolerance of plants. Regulating intercellular ABA transport may represent a novel strategy for breeding stress-tolerant plants.

11.3 Phosphorylation Networks in Cellular Signal Transduction of Stress Responses

11.3.1 Phosphorylation in Abiotic Stress Signal Transmission in Plant Cell

11.3.1.1 Roles of SnRK2 Protein Kinases in Abiotic Stress Signaling and Resistance

Dehydration-induced ABA accumulation is important for regulating stress responses in various tissues. Genetic screening of mutants showing altered ABA responses uncovered several factors that encode protein kinases and protein phosphatases. This implies the importance of phosphorylation signals in ABA responses. SNF1-related protein kinase 2 (SnRK2s) proteins are major protein kinases that transmit the ABA signal to whole plant cells. SnRK2s consist of a family of ten members in the *Arabidopsis* genome and can be classified into three subclasses. Among them, subclass III SnRK2s are mainly activated by ABA treatment, and they phosphorylate a wide variety of proteins in response to ABA (Boudsocq et al. 2004). The investigation of ABA sensing modules that consist of PYR/PYL/RCAR, protein phosphatase 2Cs (PP2Cs), and SnRK2s advanced our knowledge of drought stress responses through phosphorylation signals (Cutler et al. 2010; Raghavendra et al. 2010; Umezawa et al. 2010).

Subclass III SnRK2s play essential roles in ABA responses, including seed dormancy, stomatal closure, ABA-mediated gene expressions, and drought stress resistance (Umezawa et al. 2010). ABA-responsive element-binding factors (AREBs/ABFs), ABA-insensitive 3 (ABI3), and ABI5 are targets of SnRK2s (Kobayashi et al. 2005; Furihata et al. 2006; Sirichandra et al. 2010). These SnRK2s-AREBs/ABFs signals regulate most ABA responses, such as ABA-mediated gene expression and physiological responses (Sect. 11.3.1).

A recent report showed that SnRK2s phosphorylate and inactivate SWI/SNF chromatin-remodeling ATPase BRAHMA (BRM) (Peirats-Llobet et al. 2016). The inactive form of BRM mediates ABI5 expression. It has been proposed that SnRK2s-mediated ABA signaling mediates chromatin remodeling (Han et al. 2012). Brassinosteroid (BR)-insensitive 2 (BIN2) is a glycogen synthase kinase 3 (GKS3)-like kinase and regulates the activity, stability, and subcellular localization of its target proteins (Grimes and Jope 2001; Saidi et al. 2012). BIN2 phosphorylates SnRK2s and enhances their kinase activities in response to ABA (Yan et al. 2009; Cai et al. 2014). These studies imply a convergent pathway affecting both ABA and BR signaling.

Recently, ABA and abiotic stress-responsive Raf-like kinase (ARK) were identified as regulators of SnRK2s in *Physcomitrella patens* (Saruhashi et al. 2015). ARK belongs to a group B3 Raf-like mitogen-activated protein kinase kinase kinase (B3-MAPKKK). ARK interacts with and phosphorylates SnRK2s in response to ABA. ARK-mediated phosphorylation status controls SnRK2 activity, indicating that MAPKs directly regulate SnRK2 signaling in moss. There are six B3-MAPKKK genes that are homologs of ARK in the *Arabidopsis* genome. Further analyses of *Arabidopsis* B3-MAPKKK are expected to elucidate the crosstalk between SnRK2s and MAPKs in ABA signaling.

Subclass I family SnRK2s are activated by osmotic stress, but not by ABA. Subclass I SnRK2s and their downstream substrate,

VARICOSE (VCS), an mRNA decapping activator, have recently been shown to regulate mRNA decay under osmotic stress conditions (Soma et al. 2017). The posttranscriptional regulation mediated by the activated VARICOSE represents a novel regulatory mechanism of gene expression that facilitates drastic changes in the mRNA population under osmotic stress. Subclass I-type SnRK2s are found in seed plants but not in lycophytes or mosses. This may increase the adaptability of seed plants to stress conditions during evolution.

11.3.1.2 Histidine Kinases and Other Membrane Components in Osmotic Stress Sensing

The two-component regulatory system (the His-Asp phosphorelay) is widely involved in responses to various abiotic stresses in several organisms. In this system, histidine kinase (HK) functions in perceiving environmental stresses, including osmotic stress. In *Arabidopsis*, there are eight HK genes. Among them, two HKs (ETR1 and ERS1) are involved in ethylene perception, and three HKs (AHK2, AHK3, and AHK4) are involved in cytokinin perception. Among the remaining HKs, AHK1 (initially named ATHK1) was identified as an osmosensor and a positive regulatory factor in the osmotic stress response (Fig. 11.2). AHK1 complements a yeast *sln1* mutant for an osmosensor SLN1-encoding HK. AHK1 functions as an osmosensor in yeast by activating the downstream HOG1 MAPK cascade (Urao et al. 1999). Overexpression of AHK1 in *Arabidopsis* plants increased osmotic stress tolerance, but the *ahk1* knockout mutant showed a stress-sensitive phenotype. These results suggest that AHK1 can function as a posi-

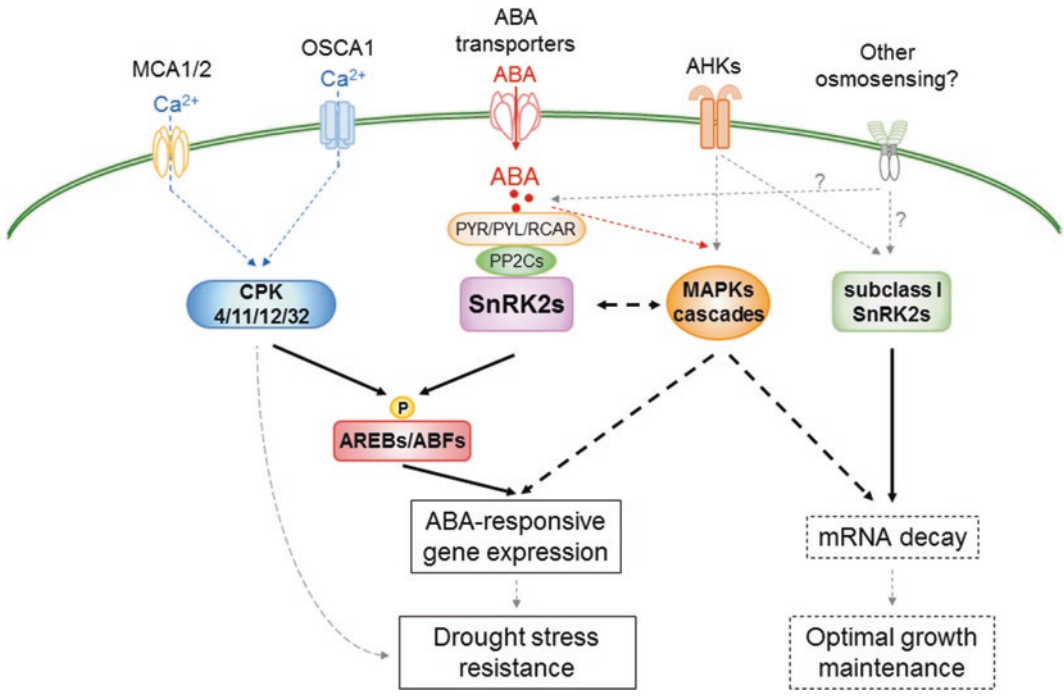

Fig. 11.2 Phosphorylation networks in plant cells in response to drought stress. Various plasma membrane proteins, such as ABA transporters, OSCA1, MCAs, and AHKs, transmit abiotic stress signals into plant cells. Protein kinases, including SnRK2s, MAPKs, and CDPKs, regulate the protein phosphorylation network in dehydration and/or ABA signaling. Subclass III SnRK2s are core components of ABA responses, and phosphorylate AREB/ABF transcription factors to regulate ABA-responsive gene expression. These SnRK2-AREB/ABF signals also mediate the drought stress resistance phenotype. MAPKs and CDPKs are other protein kinases that mediate phosphorylation signals in response to drought stress. Subclass I SnRK2s maintain mRNA decay to adjust optimal growth under abiotic stress conditions

tive regulator in osmosensing and stress tolerance (Tran et al. 2007). Other AHKs involved in cytokinin signaling (AHK2, AHK3, and AHK4) have been reported to function as negative regulators of osmotic stress responses. These studies revealed a crosstalk between osmotic stress response and cytokinin regulation (Jeon et al. 2010; Nishiyama et al. 2011).

Calcium (Ca^{2+}) is a well-known second messenger in response to abiotic stress. The Ca^{2+} oscillation in cytosolic space is transmitted into intracellular through plasma membrane proteins and Ca^{2+} channels. Reduced hyperosmolality-induced Ca^{2+} increase 1 (OSCA1) was identified as a plasma membrane protein mediating osmotic stress responses based on ethyl methanesulfonate (EMS) mutagenesis screening (Yuan et al. 2014). The *osca1* mutant shows the repression of rapid osmotic stress-induced Ca^{2+} accumulation in whole plant cells. In addition, OSCA1 functions as an upstream factor to ABA-mediated stomatal closure in guard cells. Therefore, rapid Ca^{2+} influx mediates early responses of stomatal closure before synthesis and/or transport of ABA into guard cells under stress conditions.

The Ca^{2+}-permeable mechanosensitive channel 1 (MCA1) and MCA2 are other Ca^{2+} uptake channels that mediate plant growth (Nakagawa et al. 2007; Yamanaka et al. 2010). MCA1 functionally complemented the lethal phenotype of the *mid1* yeast mutant, indicating MCA1 acts as a sensor of cell wall tension.

11.3.1.3 Potentiating Effects of MAPK and Calcium Signaling in Abiotic Stress Responses

Mitogen-activated protein kinase (MAPK/MPK) cascades are other components of phosphorylation signals in response to drought stress and ABA treatment. It has been proposed that MAPK cascades may mediate phosphorylation signals downstream of HKs in response to abiotic stress conditions, although direct interaction between HKs and MAPKs has yet to be shown. Recent studies indicate that MAPK kinase 3 (MKK3) mediates several stress responses, including ABA, jasmonic acid, and ROS signaling (Takahashi et al. 2007, 2011; Danquah et al. 2015). The triple MAPK cascade component, MAPKKK17/18-MKK3-MPK1/2/7/14, mediates ABA and dehydration stress signaling (Danquah et al. 2015; Li et al. 2017). This MAPK complex regulates stomatal closure, ABA- and/or drought-induced gene expressions, and drought stress resistance. ABA-insensitive 1 (ABI1) that encodes PP2C interacts with MAPKKK18 to regulate its kinase activity. The MAPKKK18-ABI1 module mediates the ubiquitin-proteasome pathway in response to ABA signal to maintain protein stability (Mitula et al. 2015). MAPKKK18 also mediates ABA-induced leaf senescence (Matsuoka et al. 2015). The MKK3-MPK1/2/7 module is the direct downstream cascade of MAPKKK18 in this physiological function of ABA.

The multigene families of calcineurin B-like proteins (CBLs), CBL-interacting protein kinases (CIPKs), and Ca^{2+}-dependent protein kinases (CDPKs/CPKs) encode calcium sensor protein kinases to decode intracellular Ca^{2+} transients (Hamel et al. 2014). Those proteins have specific structure domain with the EF-hand motif to capture Ca^{2+}, and the binding of Ca^{2+} induces conformational changes to activate signal transduction pathways. CPK4, CPK11, CPK12, and CPK32 phosphorylate AREBs/ABFs transcription factors that are major regulators of ABA-responsive gene expression (Choi et al. 2005; Zhu et al. 2007; Zhao et al. 2011). Phosphoproteomics analysis revealed that SnRK2s and CDPKs/CPKs share some substrates in ABA signaling. They have common phosphorylation target motif [R/K--x-x-pS/pT] (Sebastia et al. 2004; Klimecka and Muszynska 2007; Umezawa et al. 2013). These results suggest that CDPKs/CPKs act as alternative components of the ABA signaling complex in stomatal responses.

11.3.1.4 Adjustment of Optimal Growth Maintenance Under Mild Stress Conditions

Recent studies revealed novel substrates of protein kinases that mediate the adjustment of mRNA abundance. The regulation of mRNA turnover contributes to developmental processes and stress responses, but not stress resistance,

suggesting that mRNA decay machinery is an additional important response to mediate optimal growth under stress conditions (Xu et al. 2006; Goeres et al. 2007; Xu and Chua 2011; Maldonado-Bonilla 2014). A phosphoproteomics analysis showed that Decapping 2 (DCP2) phosphorylation increases in response to osmotic stress (Stecker et al. 2014). DCP1, another member of the decapping proteins, is a phosphorylation substrate of MPK6 that acts in response to drought stress, and then phosphorylated DCP1 preferentially interacts with DCP5 and promotes mRNA decapping (Xu and Chua 2012). SM-like protein 1 (LSM1) is another component of mRNA decay that promotes decapping machinery. LSM1 has a consensus phosphorylation motif of MAPKs in its protein sequence (Perea-Resa et al. 2016). Interestingly, the LSM1 complex directly regulates mRNA stability of *9-cis-epoxycarotenoid dioxygenase* (*NCED*) genes that are key enzymes of ABA biosynthesis under abiotic stress conditions. This finding implies a relationship among MAPK signaling, ABA biosynthesis, and mRNA decay machinery. It remains unclear whether the activity of mRNA decay components depends on their phosphorylation status. Further analyses are required to understand their detailed mechanisms.

11.3.2 Signal Transduction in Guard Cells

11.3.2.1 ABA-Mediated Stomatal Control via SnRK2 Protein Kinases

Plant stomatal movement plays a key role in maintaining water homeostasis and in modulating CO_2 availability from photosynthesis. Several studies have suggested that phosphorylation signals play major roles in stomatal aperture, although detailed signal cascades in guard cells are still unclear (Desikan et al. 2004; Asano et al. 2012; Liu 2012).

Several ion channels have been shown to be substrates of SnRK2s in guard cells (Fig. 11.3). An S-type anion channel, slow anion channel 1 (SLAC1), regulates anion efflux for an essential step of stomatal closure in response to ABA (Negi et al. 2008; Vahisalu et al. 2008). SnRK2s phosphorylate SLAC1 in an ABA-dependent manner (Geiger et al. 2009; Lee et al. 2009; Brandt et al. 2012). Recent studies indicate that SnRK2s interact with and activate SLAC1 homolog 3 (SLAH3) and quickly-activating anion channel (QUAC1) in response to ABA (Geiger et al. 2009; Imes et al. 2013). Potassium (K^+) channels in *A. thaliana* (KAT1) are additional substrates for SnRK2s in guard cells. SnRK2s phosphorylate KAT1 and inhibit its channel activity to prevent stomatal opening via K^+ influx (Sato et al. 2009).

Transcription factors are also targets of SnRK2s phosphorylation. ABA-responsive kinase substrate 1 (AKS1), AKS2, and AKS3 have been identified as the interaction partners of 14–3–3 protein in guard cells (Takahashi et al. 2013). AKSs are basic helix-loop-helix (bHLH) transcription factors and control stomatal opening in response to blue light. SnRK2s phosphorylate AKSs and inhibit transcriptional activity of AKSs in response to ABA treatment. AKSs directly regulate the expression of the *KAT1* gene. A current model has been proposed that ABA-activated SnRK2s phosphorylate AKSs to repress their transcriptional activity, and then the spate of responses cause the inhibition of K^+ influx-mediated stomatal opening to promote stomatal closure in response to ABA.

Recent studies have shown that NADPH oxidase, respiratory burst oxidase homolog D/F (RbohD/F), and K^+ uptake transporter 6/8 (KUP6/8) are also regulated by SnRK2s (Sato et al. 2009; Sirichandra et al. 2009; Acharya et al. 2013; Osakabe et al. 2013). SnRK2s can phosphorylate a wide variety of substrates in ABA-mediated stomatal responses. Guard cell-specific phosphoproteomics analysis should reveal how SnRK2 regulates the phosphorylation network in guard cells.

11.3.2.2 MAPK Signaling in Stomatal Responses

Guard cell-specific transcriptome analyses indicated that several MAPKs mediate stomatal control in response to ABA treatment (Wang et al.

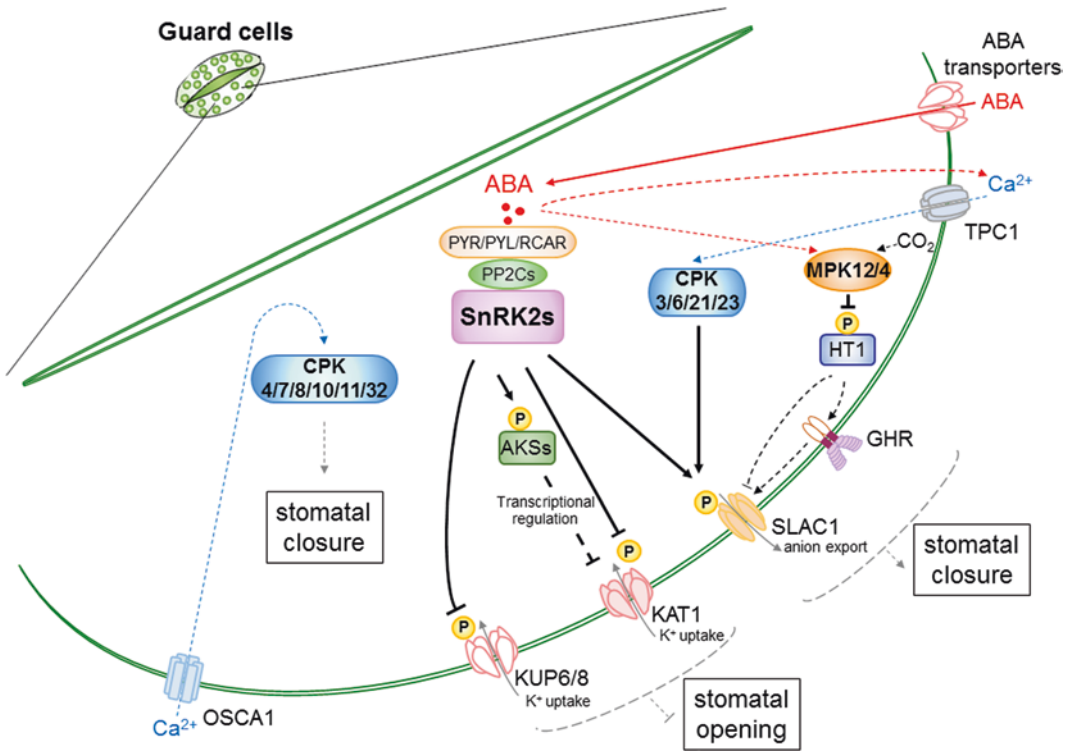

Fig. 11.3 Stomatal control via SnRK2s, MAPKs, and CDPKs in response to drought stress. Subclass III SnRK2s phosphorylate a wide variety of proteins, such as the ion channels SLAC1, SLAH3, QUAC1, KAT1, or KUP6/8, the transcription factors of the AKS family, and the NADPH oxidase Rboh D/F. CDPKs also phosphorylate SLAC1 to mediate stomatal closure in response to ABA and/or Ca^{2+} accumulation. MAPKs are activated by ABA or CO_2 accumulation, and they regulate SLAC1 activity in association with HT1 or GHR in guard cells

2011). This approach revealed that MPK9 and MPK12 function as upstream factors of anion channels and regulate stomatal closure in response to ABA treatment (Jammes et al. 2009). Quantitative trait locus (QTL) mapping was used to identify key factors mediating WUE (Des Marais et al. 2014). Based on QTL screening of an *Arabidopsis* Landsberg erecta (Ler) × Cape Verde Island (Cvi) mapping population, MPK12 was isolated as a signal mediator to regulate stomatal conductance. MPK12 mediates ABA-induced stomatal closure, guard cell size, and water transpiration from leaves.

Recent studies have reported that MPK12 and MPK4, which belong to the same subgroup of MPK12, interact with another kinase, high leaf temperature 1 (HT1) (Horak et al. 2016; Jakobson et al. 2016). This MPK4/MPK12-HT1 signaling module regulates the activity of SLAC1 to inhibit stomatal opening under high CO_2 concentrations. The MPK12-HT1 module regulates the activity of guard cell hydrogen peroxide-resistant 1 (GHR1) as another downstream factor. GHR1 is known to function as a receptor-like kinase localized on the plasma membrane in guard cells and an upstream factor of SLAC1 (Hua et al. 2012; Horak et al. 2016). Taken together, the MPK4/MPK12-HT1 module controls CO_2-induced stomatal closure via regulation of SLAC1 activity. Further studies will uncover coordinated and/or parallel signaling of stomatal aperture in response to ABA and CO_2, and this knowledge will improve WUE in a shifting global climate.

11.3.2.3 Calcium Influx and Ca^{2+}-Regulated Protein Kinases Signaling in Guard Cells

The slow vacuolar channel is a Ca^{2+} transporter that mediates sustainable Ca^{2+} influx. *Arabidopsis* whole-genome annotation analysis revealed that the slow vacuolar channel is encoded as a single gene, *two-pore channel 1* (*TPC1*), which mediates a Ca^{2+}-induced Ca^{2+} release mechanism (Ward and Schroeder 1994; Peiter et al. 2005). The *tpc1* mutant does not show stomatal closure in response to treatment of high Ca^{2+} concentration, even though ABA treatment enhances stomatal closure in the *tpc1* mutant. It has been reported that ABA provokes Ca^{2+} oscillation in plant cells (Kim et al. 2010). Taken together, an elevated Ca^{2+} accumulation is required to maintain stomatal closure for an extended period of time after ABA generates guard cell responses under stressful conditions.

The CBL-CIPK modules act downstream of Ca^{2+} influx in guard cells. CBL1 and CBL9 interact with CIPK23 and mediate stomatal aperture in response to ABA treatment (Cheong et al. 2007). CBL1-CIPK1 is another CBL-CIPK interacting module. The dissociation of CBL1 and CIPK1 is enhanced in response to cytosolic Ca^{2+} concentration, and the released CIPK1 mediates mitochondrial functions in guard cells (Tominaga et al. 2010).

In guard cells, CDPKs/CPKs mainly regulate ion channels to regulate stomatal closure in association with ABA signaling. CPK3 and CPK6 regulate S-type anion and Ca^{2+}-permeable channels in response to ABA (Mori et al. 2006). CPK6, CPK21, and CPK23 phosphorylate an anion channel of SLAC1 as the downstream pathways of ABA signaling (Geiger et al. 2010; Brandt et al. 2012). Interestingly, recent studies show another aspect of CDPKs/CPKs for stomatal regulations. CPK10, CPK4/CPK11, and CPK7/CPK8/CPK32 mediate stomatal closure in response to Ca^{2+} oscillation even though these protein kinases typically mediate ABA-induced stomatal closure (Hubbard et al. 2012). CDPK/CPK signaling will be partially independent on ABA signaling in stomatal responses, and further studies will be required to explain how CDPKs/CPKs have influenced ABA signaling in guard cells.

11.4 Gene Expression Regulatory Networks

In plants, the most important targets activated downstream of stress signal cascades are various transcriptional regulatory factors. Many previous studies have revealed drought stress-responsive genes that have critical roles for response and adaptation to drought stress in plants (Seki et al. 2002; Maruyama et al. 2012; Urano et al. 2017). Transcriptional regulation is more important in plants than animals, because there are more transcription factors and more variation in the number of transcription factors in plants compared to animals.

11.4.1 Transcription Factors Regulating Gene Expression During Drought Stress

11.4.1.1 bZIP Transcription Factor Family

An ABA-responsive element (ABRE, PyACGTGGC) was identified as an enriched motif on promoters of drought stress-responsive genes (Maruyama et al. 2012). bZIP-type transcription factors ABRE-binding proteins (AREBs)/ABRE-binding factors (ABFs) bind to this motif and activate their respective target genes (Fig. 11.4). Nine subfamily members of AREBs/ABFs share a bZIP domain and a conserved domain containing Ser/Thr kinase phosphorylation sites, with three members among them (AREB1/ABF2, AREB2/ABF4 and ABF3) that show drought stress inducibility in *Arabidopsis* (Fujita et al. 2005). In an ABA signaling pathway, subclass III SnRK2s (SRK2D/SnRK2.2, SRK2E/SnRK2.6, and SRK2I/SnRK2.3) phosphorylate the target sites and activate the transcriptional activity of AREBs/ABFs

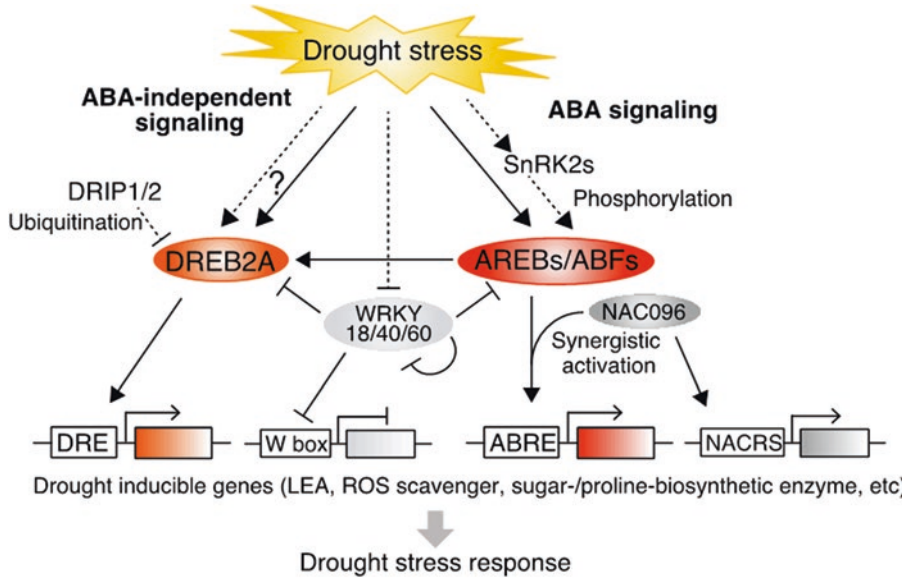

Fig. 11.4 A schematic model of a transcriptional and posttranscriptional regulatory network in the drought stress response. The AREB/ABF proteins are activated by SnRK2s through phosphorylation in the ABA-dependent signaling, and the activated ABREs/ABFs induce drought stress-inducible genes through their binding element ABRE during drought stress, and the *AREBs/ABFs* genes are induced by drought stress. The DREB2A protein is unstable under non-stressful conditions through ubiquitination by DRIP1/2, and it is suggested that unknown mechanisms in the ABA-independent signaling induce the *DREB2A* gene and stabilize the DREB2A protein. The accumulated DREB2A protein activates its target genes through the binding element DRE. The AREBs/ABFs have also been reported to activate the *DREB2A* gene through ABA-dependent signaling. WRKY18/40/60 repress the expression of drought stress-inducible genes, including *AREBs/ABFs* and *DREB2A*, through the W box binding element, and they repress the expression of each other. The WRKY18/40/60 proteins are exported from nuclei during drought stress to decrease the negative effects on their target genes. NAC096 interacts with AREBs/ABFs and activates its target genes synergistically through the binding element NACRS. These transcription factors regulate their specific or common target genes and induce drought stress responses in plants. Solid lines indicate regulation of gene expression, and dashed lines indicate regulation of protein activation

(Fujita et al. 2009). Amino acid substitutions in the phosphorylation sites that mimic Asp enhanced the transcriptional activity of AREB1 in protoplasts, even without ABA treatment (Furihata et al. 2006). Expression levels of many drought stress-inducible genes were suppressed during dehydration stress in triple mutants of AREB1, AREB2, and ABF3, and these triple mutants showed drought stress-sensitive phenotypes (Yoshida et al. 2010). On the promoters of ABA-responsive genes, ABRE motifs often co-locate with coupling element 3 (CE3) (Shen et al. 1996). However, which factors bind to this motif and function together with AREBs/ABFs is still unknown.

11.4.1.2 AP2/ERF (APETALA2/Ethylene-Responsive-Binding Factor) Family

Dehydration-responsive element (DRE, A/GCCGAC) is another common motif found in the promoters of drought stress-responsive genes (Maruyama et al. 2012), and DRE-binding protein 2A (DREB2A) was identified as a binding factor of the DRE motif (Liu et al. 1998). The DREB2A gene is induced by AREBs/ABFs under dehydration stress conditions. However, unidentified factors in ABA-independent signaling are also involved in the induction of DREB2A during drought stress response (Kim et al. 2011). Overexpression of the wild-type form of

DREB2A does not affect the expression of drought stress-inducible genes (Sakuma et al. 2006a). The reason is that the DREB2A protein is destabilized and degraded through a negative regulatory domain (NRD) under non-stressful conditions. Constitutively active forms of DREB2A (DREB2A CA) that lack the NRD are stabilized and activate target genes even under non-stressful conditions. DREB2A-interacting protein 1 (DRIP1) and DRIP2 are C3H4 RING domain-containing proteins that have been reported to degrade the DREB2A protein through the 26S proteasome pathway under non-stressful conditions (Qin et al. 2008). However, the activating mechanisms that stabilize the DREB2A protein during stress remain to be discovered. Moreover, DREB2A activates heat stress-inducible genes under heat stress conditions. Therefore, this transcription factor functions in the crosstalk between drought and heat stress signaling (Sakuma et al. 2006b). It has been reported that a heat stress-specific transcriptional complex activates the transcriptional activity of DREB2A during heat stress (Sato et al. 2014). However, drought stress-specific transcriptional coactivators have yet to be identified.

11.4.1.3 WRKY Family

WRKY transcription factors bind to the W box (TTGACC/T), and there are detailed studies about WRKYs showing negative effects on ABA signaling (Rushton et al. 2012). WRKY18, WRKY40, and WRKY60 directly bind to promoters of many ABA-inducible genes (e.g., ABI4, ABI5, ABF4, DREB2A) that suppress the expression of those target genes under non-stressful conditions (Chen et al. 2010; Shang et al. 2010). These three WRKY factors also negatively affect each other's gene expression (Yan et al. 2013). Triple mutants of WRKY18, WRKY40, and WRKY60 showed an ABA hypersensitive phenotype. Under drought stress conditions, these three WRKYs are exported from nuclei and interact with magnesium-protoporphyrin IX chelatase H subunit (CHLH/ABAR) in the chloroplast to decrease the negative effect on the drought stress-inducible target genes (Shang et al. 2010). There are also some WRKY transcription factors that positively regulate drought stress responses. ABA overly sensitive 3 (ABO3)/WRKY63 is induced by ABA treatment, and the knockout mutants showed drought stress-sensitive phenotypes. Subsequent studies revealed that this factor was bound directly to the promoters of some drought stress-inducible genes, such as ABF2, and activated gene expression during drought stress (Ren et al. 2010).

11.4.1.4 NAC (NAM-ATAF1,2-CUC2) Family

There are several NAC genes induced by drought stress or ABA treatment that are involved in the regulation of gene expression during drought stress through their consensus NAC recognition site (NACRS, CGTG/A) (Puranik et al. 2012). ANAC096 is a drought stress-inducible NAC gene and is thought to activate many drought stress-inducible genes. This factor interacted with AREB1/ABF2 and AREB2/ABF4 and synergistically activated the RD29A gene in protoplasts. Knockout mutants of ANAC096 showed a decreased drought stress tolerance (Xu et al. 2013b). On the other hand, ANAC016 is induced by drought stress, but it has negative effects on expression levels of drought stress-inducible genes, including AREB1 (Sakuraba et al. 2015). Therefore, the knockout of NAC016 increased expression levels of drought stress-inducible genes and drought stress tolerance. Some drought-inducible NAC proteins are also reported to regulate ABA-dependent leaf senescence under drought stress conditions. Septuple mutants of the A subfamily of drought inducible-NAC (ANAC055, ANAC019, ANAC072/RD26, ANAC002/ATAF1, ANAC081/ATAF2, ANAC102, and ANAC032) showed delayed leaf senescence by ABA treatment. Microarray analysis revealed that many senescence-inducible genes were downregulated in this septuple mutant (Takasaki et al. 2015).

11.4.1.5 Other Transcription Factor Families

There are still other transcription factor families that regulate transcriptomic changes under

drought stress conditions in *Arabidopsis* (Dubos et al. 2010; Feller et al. 2011; Harris et al. 2011). Homeobox protein 6 (HB6) that belongs to a homeodomain-leucine zipper (HD-Zip) transcription factor family is suggested to negatively regulate the ABA response, because overexpression of *HB6* results in insensitive phenotypes in response to ABA treatment (Himmelbach et al. 2002). A MYB domain-containing transcription factor MYB96 is induced by drought stress and has been suggested to function in the crosstalk between ABA and auxin (Seo et al. 2009). In conjunction with MYB2, a basic helix-loop-helix (bHLH)-containing transcription factor, MYC2, positively regulates ABA signaling (Abe et al. 2003). Future studies will reveal the complicated transcriptional network comprising these factors under drought stress conditions.

11.4.2 Posttranscriptional Regulation by Small RNAs During Drought Stress

Small noncoding RNAs, such as microRNAs (miRNAs) and short interfering RNAs (siRNAs), function as negative regulators of gene expression at posttranscriptional levels by guiding target mRNAs for degradation or by repressing their translation (Sunkar et al. 2007). Transcriptome analyses have identified that expression levels of many small RNAs are affected by drought stress, suggesting that these small RNAs are involved in drought stress responses (Sunkar and Zhu 2004; Zhou et al. 2010).

The *miR169a* targets the mRNA of nuclear factor Y subunit A5 (NF-YA5), and drought stress suppresses the expression level of miR169a (Zhao et al. 2009). Downregulation of *miR169a* expression is suggested to enhance the accumulation of the NF-YA5 mRNA during drought stress. These mechanisms are important for drought stress responses in plants, because the knockout of *NF-YA5* or overexpression of *miR169a* decreased drought stress tolerance (Li et al. 2008). *miR159* is induced by ABI3 under drought stress conditions and targets the mRNA of MYB33 and MYB101 transcription factors that are positive regulators of drought stress responses. Overexpression of *miR159* resulted in insensitive phenotypes to ABA treatment during germination. Therefore, this small RNA is suggested to control homeostasis of drought stress responses (Reyes and Chua 2007).

Small RNAs are also involved in affecting plant architecture under drought stress conditions. IAA-Ala Resistant3 (IAR3) was identified as a cleavage target of *miR167a* by computer analysis. Drought stress suppressed the expression of *miR167a* and consistently led to accumulation of *IAR3* mRNA. The IAR3 protein is involved in auxin biosynthesis, as well as primary and lateral root growth during osmotic stress. These root architecture changes are important for drought stress tolerance in plants, because the knockout of *IAR3* significantly decreased drought stress tolerance (Kinoshita et al. 2012).

11.4.3 Epigenetic Regulation During Drought Stress

More and more evidences suggest that epigenetic regulation, such as DNA methylation and histone modification, play key roles in transcriptional regulation of several genes in response to drought stress (Chinnusamy and Zhu 2009). Genome-wide and gene-specific analyses revealed that DNA methylation and histone modification, such as histone 3 lysine 4 trimethylation (H3K4 me3) and histone 3 lysine 9 acetylation (H3K9ac), are modified during drought stress conditions (van Dijk et al. 2010; Kim et al. 2012; Colaneri and Jones 2013).

Arabidopsis Homologue of Trithorax1 (ATX1) is a histone modification enzyme that trimethylates histone H3 at lysine 4. Knockout mutants of *ATX1* showed larger stomatal aperture phenotypes during drought stress and decreased drought stress tolerance in *Arabidopsis* (Ding et al. 2011). This factor directly binds to a gene encoding an ABA biosynthetic enzyme, and trimethylation of H3K4 during drought stress is suggested to be involved in recruitment of RNA

polymerase II on the promoter. Histone deacetylase 9 (HDA9) is another histone modification enzyme that regulates transcriptional changes during drought stress. Knockout mutants of *HDA9* resulted in the enhancement of H3K9ac on the promoter of many drought stress-inducible genes and showed enhanced expression levels of those genes (Zheng et al. 2016). Moreover, these knockout mutants are hypersensitive to osmotic stress. Therefore, this factor is thought to be a negative regulator of drought stress responses through epigenetic regulations. Epigenetic regulation of drought stress-inducible genes is also involved in other aspects of plant hormone signaling. Knockout mutants of histone deacetylase 6 (HDA6) were recently identified to show enhanced drought stress tolerance (Kim et al. 2017). Transcriptome analyses revealed that drought-inducible genes in an acetate biosynthetic pathway were upregulated in these mutants, and acetic acid promoted jasmonate signaling in drought stress responses. The analysis of the *hda6* mutants suggested that acetate-inducible jasmonate signaling was independent of ABA signaling. On the other hand, other study also was reported that the exogenous methyl jasmonate induced stomatal closure through ABA pathway (Yin et al. 2016). There is still much to be learned about the detailed molecular mechanisms in the crosstalk of ABA and jasmonate during drought stress.

Recent studies suggest that the epigenetic regulation of drought stress-responsive genes also play important roles in "stress memory" to prepare the secondary stress responses after the first stress exposure. Some genes that were hyper-induced by repeated drought stress conditions were identified as "trainable genes." This hyper-responsiveness to the repeated drought stress is suggested to be associated with some epigenetic markers, such as H3K4me3 or histone 3 lysine 3 trimethylation (H3K27me3) (Ding et al. 2012; Liu et al. 2014). ChIP-qPCR assays revealed that active RNA polymerase II with phosphorylated serine 2 remains on the promoters of trainable genes even after the first stress treatment. It is critically needed to learn about the molecular mechanisms in which drought stress generates, maintains, and deletes stress memory.

11.4.4 Drought Stress-Inducible Genes Encoding Other Functional Proteins

The transcription factors, small RNAs, and epigenetic regulators described above control expression levels of specific or common target genes during drought stress. Previous studies revealed the molecular functions of some enzymatic or chaperone-like proteins encoded by those target genes. These proteins work to maintain cellular conditions or sustain other protein functions during drought stress. Some examples are described below.

11.4.4.1 Late Embryo Abundant (LEA) Proteins

LEA proteins are typical drought stress-responsive factors and have been found to accumulate in the late stage of seed development in plants. Subsequent studies have revealed that genes encoding these factors also are induced by drought stress at vegetative stages and that microbes and invertebrates contain homologous genes in response to drought stress (Hincha and Thalhammer 2012). There is still much to be elucidated regarding the detailed molecular functions of the LEA proteins. However, some studies have suggested that these proteins could function like molecular chaperones. For example, an LEA family protein in wheat prevented protein aggregation due to desiccation in vitro (Goyal et al. 2005). Knockout of Low Temperature-Induced 30 (LTI30), a group II LEA family gene, resulted in decreased drought stress tolerance in *Arabidopsis* (Shi et al. 2015).

11.4.4.2 Reactive Oxygen Species (ROS) Scavenging Enzymes

Reactive oxygen species (ROS), such as $^1O_2$, H_2O_2 and O^{2-}, are produced upon drought stress treatments and are thought to function as secondary messengers in drought stress signaling (Miller

et al. 2010). Meanwhile, overproduction of ROS causes oxidative damage to cellular components. Several ROS-scavenging enzymes, such as superoxide (SOD) and ascorbate peroxidase (APX), are induced by drought, and these proteins convert ROS into nontoxic molecules to prevent ROS overaccumulation. ROS signaling also plays an important role in stomatal cells. Knockout mutants of *Arabidopsis* glutathione peroxidase (ATGPX) led to overaccumulation of ROS in stomata during ABA treatment and decreased drought stress tolerance due to the higher water loss (Miao et al. 2006).

11.4.4.3 Sugar- or Proline-Biosynthetic Enzymes

Several sugars, such as galactinol, trehalose, and fructan, as well as the amino acid proline, accumulate under drought stress conditions and are thought to function as osmolytes to maintain cell turgor and protein structures (Seki et al. 2007). Recently, other functions of these osmolytes have been reported, including ROS scavenging (Nishizawa et al. 2008), electron flow (Chaves et al. 2009), and reproductive development (Mattioli et al. 2009). Their detailed molecular functions have yet to be elucidated. Galactinol synthase 2 (GolS2) is an enzyme producing galactinol that is an oligosaccharide. The *GolS2* gene is expressed in response to drought stress, and overexpression of *GolS2* gene leads to a drought stress resistant phenotype in *Arabidopsis* and *Brachypodium* (Taji et al. 2002; Himuro et al. 2014). Recently, we reported that Brazilian and African rice overexpressing the *GolS2* gene showed higher yield, greater biomass, and drought stress resistance than control rice over a 3-year period in different dried fields (Selvaraj et al. 2017).

11.5 Conclusions and Future Perspectives

In this review, we have summarized recent advances of drought stress signaling, focusing on gene networks associated with ABA-related cellular and intercellular responses under drought stress conditions. The phytohormone, ABA, is a key player that regulates various cellular and intercellular responses, including avoidance, adaptation, and resistance to drought stress. We discussed how plants initiate ABA biosynthesis and transport ABA to the appropriate tissues in response to drought stress. Recent studies indicate that ABA is synthesized mainly in leaves in response to drought stress. These results imply that mobile molecules mediate the initiation of ABA biosynthesis in root-to-shoot communications. It has been proposed that several mobile signaling mechanisms, such as hydraulic pressure, Ca^{2+} oscillation, or ROS, move from roots to shoots under abiotic stress conditions. A change in the water potential in the roots creates water vapor gradients, leading to the regulation of transpiration by stomatal closure (Christmann et al. 2007). Cytoplasmic Ca^{2+} concentrations drastically change from roots to shoots when plant root tips were exposed to various abiotic stress conditions, such as cold, ROS, salinity, and physical stimuli (Choi et al. 2014). However, no long-distance signaling molecules that can trigger ABA accumulation in leaves had been identified. Recently, we identified one of the peptides that links the sensing of drought stress in roots and ABA accumulation in leaves (unpublished data). Further analyses are required to elucidate this missing link between the perception of drought stress conditions in roots and the initiation of ABA biosynthesis in leaves through mobile molecules.

Comparative expression analyses between ABA biosynthesis enzymes and ABA transporters are important for determining how ABA is distributed among intercellular spaces. It has been proposed that most ABA biosynthesis enzymes and ABA transporters are expressed in the vascular tissues, implying that ABA is transported from vascular veins to peripheral tissues, including stomata. Identification and analyses of ABA transporters can shed light on the active ABA influx and/or efflux machinery at the tissue level. It remains unclear whether transporter activity depends on dehydration stress conditions

and how ABA is distributed appropriately among compartmentalized cell structures, such as guard cells and mesophyll cells. Advances in visualization technology will help our understanding of ABA migration among intercellular spaces.

Phosphoproteomics has been developed as a powerful new tool to identify novel phosphorylation networks. This "omics" approach clarified the upstream and downstream substrates of SnRK2s in ABA signaling. Recent studies showed direct interaction between SnRK2s, MAPKs, and BIN2. These interactions influence kinase activity in response to ABA treatment, suggesting that protein kinases synergistically transmit their phosphorylation signals in comparison with conventional knowledge that each phosphorylation relay is performed in parallel by each protein kinase. In addition, BIN2-mediated regulation of SnRK2s activity indicates novel crosstalk that BR-induced optimal growth opposes physiological effects of ABA-mediated growth retardation in response to dehydration stress. It is likely that protein kinases are good candidates for the crosstalk and node points. The signal crosstalk between ABA and other phytohormones, stresses, and flowering time should receive much attention in the future.

In addition to stress-resistant responses, another form of plant physiological responses is their ability to adjust their optimal growth maintenance under mildly stressful conditions. The regulation of mRNA turnover is thought to be an important factor to modify optimal growth associated with this physiological response. Most of the control of mRNA metabolism is involved in mRNA decapping in P bodies. However, it remains unclear how the mRNA targeted for degradation relocates to P bodies and interacts with other factors including RNA-binding proteins, decapping factors, and other stress-induced proteins. The substrate specificities of the decapping machinery will also mediate the mobility and disassembly of targeted mRNA in response to stressful conditions. Further analyses of mRNA decay mechanisms are necessary to understand stress-mediated posttranscriptional regulations.

In conclusion, research of drought stress responses has begun to focus on long-distance communication involving ABA signaling, such as tissue-to-tissue and intercellular-to-intercellular. Systematic knowledge of drought stress responses will provide agriculture and biotechnology with critical information of stress-resistant mechanisms. Using this knowledge of drought stress responses will merit much further research for future crop innovation.

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Mechanism of Stomatal Closure in Plants Exposed to Drought and Cold Stress

12

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Abstract

Drought is one of the abiotic stresses which impairs the plant growth/development and restricts the yield of many crops throughout the world. Stomatal closure is a common adaptation response of plants to the onset of drought condition. Stomata are microscopic pores on the leaf epidermis, which regulate the transpiration/CO_2 uptake by leaves. Stomatal guard cells can sense various abiotic and biotic stress stimuli from the internal and external environment and respond quickly to initiate closure under unfavorable conditions. Stomata also limit the entry of pathogens into leaves, restricting their invasion. Drought is accompanied by the production and/or mobilization of the phytohormone, abscisic acid (ABA), which is well-known for its ability to induce stomatal closure. Apart from the ABA, various other factors that accumulate during drought and affect the stomatal function are plant hormones (auxins, MJ, ethylene, brassinosteroids, and cytokinins), microbial elicitors (salicylic acid, harpin, Flg 22, and chitosan), and polyamines. The role of various signaling components/secondary messengers during stomatal opening or closure has been a matter of intense investigation. Reactive oxygen species (ROS), nitric oxide (NO), cytosolic pH, and calcium are some of the well-documented signaling components during stomatal closure. The interrelationship and interactions of these signaling components such as ROS, NO, cytosolic pH, and free Ca^{2+} are quite complex and need further detailed examination.

Low temperatures can have deleterious effects on plants. However, plants evolved protection mechanisms to overcome the impact of this stress. Cold temperature inhibits stomatal opening and causes stomatal closure. Cold-acclimated plants often exhibit marked changes in their lipid composition, particularly of the membranes. Cold stress often leads to the accumulation of ABA, besides osmolytes such as glycine betaine and proline. The role of signaling components such as ROS, NO, and Ca^{2+} during cold acclimation is yet to be established, though the effects of cold stress on plant growth and development are studied extensively. The information on the mitigation processes is quite limited. We have attempted to describe consequences of drought and cold stress in plants, emphasizing stomatal closure. Several of these factors trigger signaling components

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in roots, shoots, and atmosphere, all leading to stomatal closure. A scheme is presented to show the possible signaling events and their convergence and divergence of action during stomatal closure. The possible directions for future research are discussed.

Keywords

Stomatal closure · Guard cells · Water stress · Chilling · Reactive oxygen species · ROS · Nitric oxide · NO · Cytosolic pH · Signaling components · Secondary messengers · ABA · Cytosolic free Ca^{2+} · Ion channels

Abbreviations

ABA	Abscisic acid
ABI1	Abscisic acid insensitive 1
ABI2	Abscisic acid insensitive 2
ASA/Acetyl-SA	Acetylsalicylic acid
CPKs	Calcium-dependent protein kinases
ET	Ethylene
H_2S	Hydrogen sulfide
MAPKs	Mitogen-activated protein kinases
MeSA	Methyl salicylate
MJ	Methyl jasmonate
NO	Nitric oxide
NOA	Nitric acid associated
NR	Nitrate reductase
OST1	Open stomata 1
PAs	Polyamines
QUAC	Quick anion channel
ROS	Reactive oxygen species
SA	Salicylic acid
SLAC	Slow anion channels

12.1 Importance of Stomata

Drought or insufficient water availability is a common environmental stress in not only tropical/subtropical areas but also temperate regions of our world. During drought, plants need to conserve water by limiting transpirational water loss, as soon as possible. Stomata play a key role in such regulation of transpiration. Plants often become susceptible for pathogens during drought (Pandey et al. 2017). The closure of stomata is also a component of plants' innate immunity response to protect against the pathogenic microorganisms, as wide spectrum of pathogens try to enter into the plants through these natural openings (Melotto et al. 2008). The architecture of stomatal pore is unique, formed by two specialized guard cells. These stomatal guard cells can sense and integrate various contradictory signals from the internal and external environment (Hetherington and Woodward 2003; Vavasseur and Raghavendra 2005). Stomata fine-tune the events in guard cells to achieve an appropriate physical response, to survive under the prevailing stress conditions.

The opening and closing of stomatal pores are brought about by changes in turgidity of guard cells, stomata being open when guard cells are turgid and closed when guard cells are flaccid (Willmer and Fricker 1996; Schroeder et al. 2001). When solutes accumulate, the water potential of guard cells is lowered. As a result, the steep water potential gradient drives water into the guard cells from the neighboring cells. Then guard cells become turgid, and swell in size, opening stomata. In a reversal of these events, when guard cells lose solutes, water moves out, making the guard cells flaccid leading to stomatal closure (Vavasseur and Raghavendra 2005; Acharya and Assmann 2009; Kim et al. 2010). The unique structure of the cell wall including plasma membrane, tonoplast, and cytoskeleton of guard cells is also involved in the regulation of stomatal movement (Gao et al. 2009).

Several factors (abiotic or biotic) modulate stomatal movements. Among the abiotic factors, light promotes stomatal opening. Similarly, warm temperature, high humidity, and hormones like cytokinins also are known to stimulate stomatal opening (Shimazaki et al. 2007; Kim et al. 2010; Murata et al. 2015). On the other hand, drought stress, cold temperature, high CO_2, darkness, and plant hormones like abscisic acid (ABA), ethylene (ET), or elicitors are known to induce stomatal closure (Acharya and Assmann 2009; Kim et al. 2010). Both drought and cold temperatures cause an imbalance in the water status of plants and induce the synthesis of phytohormone, abscisic acid (ABA) that promotes stomatal closure. Thus, stomatal closure is an adaptive response to drought, as well as cold.

Besides acting as a gateway for water/CO_2, stomata also have the ability to restrict the pathogen invasion, thus playing as primary barrier during innate immune system (Melotto et al. 2008; Zeng et al. 2010). Stomatal defense response is the result of contemporaneous action of many signaling components in the guard cells. There are excellent reviews, which emphasize the importance of stomatal closure during abiotic stress conditions (drought and cold stress) (Miura and Tada 2014; Lim et al. 2015; Murata et al. 2015; Saradadevi et al. 2017; Sussmilch and McAdam 2017). Our article mainly focuses on drought (water stress) and cold stress. During drought, transpiration exceeds the water uptake by plants. In contrast, while during cold stress, the capacity of water absorption slows down, and transpiration maintains in a steady state. Here we describe in detail the role of some major abiotic and biotic factor, which is implicated in stomatal closure during drought or cold stress conditions.

12.2 Effects of Drought or Cold Stress on Stomatal Development and Function

Plants try to adapt to unfavorable environment, such as drought or cold stress, in several ways. When plants are exposed to drought, besides the quick responses, such as stomatal closure, there are long-term effects such as decrease in leaf area, stomatal frequency, and accumulation of compatible solutes (Shi et al. 2014; Eremina et al. 2016). Thus, the onset of drought affects stomatal development, decreasing their frequency and promotes stomatal closure, so as to limit the transpirational water loss, through several components (Fig. 12.1). Drought exposure often leads to the modulation of phytohormonal levels in plants, particularly of ABA, called as a "stress hormone" (Finkelstein 2013; Vilela et al. 2015). The site of ABA production is either the roots or shoots, from where it is transported to the leaves (Seo and Koshiba 2011). Hydraulic signals from roots initiated during drought can be transmitted quickly to the shoot, and these can trigger stomatal closure. Similarly, an increase in the sulfate concentration in xylem tissue can also promote stomatal closure (Malcheska et al. 2017). This xylem sap under drought condition showed a rise in pH and changes in guard cell behavior observed (Wilkinson and Davies 1997). Apoplastic accumulation of ABA during drought due to change in pH can cause stomatal closure (Wilkinson 1999). A comprehensive list of signals triggered during drought which can cause stomatal closure is given in Table 12.1.

Cold stress is also a major environmental factor that limits plant growth and development, as well as stomatal behavior. Low-temperature treatment results in the decrease in stomatal opening and subsequently photosynthesis (Drew and Brazzaz 1982; Allen et al. 2000). Recent studies also suggest that stomatal responses form a common component of adaptation against drought and cold stress. ABA seems to be a central player in such alleviation of cold or drought stress (Fig. 12.2). The *ost1* (open stomata 1, deficient in Ser/Thr protein kinase) mutants show freezing hypersensitivity, whereas transgenic plants overexpressing OST1 exhibit enhanced freezing tolerance. In addition to ABA, H_2S, a gaseous signaling molecule, is involved in the regulation of various physiological and developmental processes including stomatal closure (García-Mata and Lamattina 2013). During cold stress, the accumulation of

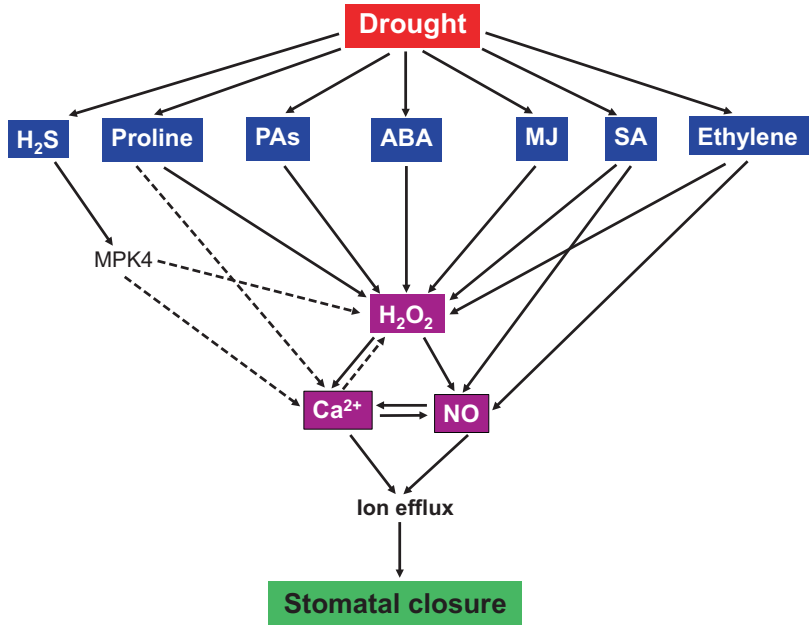

Fig. 12.1 A diagrammatic representation of hormonal/metabolite signal transduction mechanism under drought stress in stomatal guard cells during stomatal closure. Drought is often accompanied by enhancement of the plant hormones such as ABA, jasmonates, SA, ethylene, and metabolites (proline, PAs, and H_2S). The mechanism of stomatal closure induced by ABA, SA, MJ, and ethylene under drought stress is well known. In contrast, metabolites like proline, PAs, and gaseous signal such as H_2S-induced stomatal closure are obscure. ABA, SA, MJ, ethylene, and PAs caused stomatal closure through the production of signaling components such as ROS, NO, and Ca^{2+} in stomatal guard cells; these secondary messengers subsequently modulate ionic status of the guard cells which causes decrease in the turgor pressure and stomatal closure. The stimulation of reactions with the experimental evidences is represented by solid arrows. The obscure interactions or the relationships where the evidences are in haze are represented by broken arrows. The inhibition/deficiency is indicated by ⊣

H_2S upregulates mitogen-activated protein kinase 4 (MAPK4). Stomatal development is also affected leading to a decrease in the stomatal frequency under cold conditions (Hetherington and Woodward 2003; Vatén and Bergmann 2012). This would ensure a decrease in transpiration and conservation of water. Stomata can also sense the onset of drought or cold stress due to several phenomena. The stomatal closure under such conditions, a common response, is due to the accumulation of compounds such as ABA, methyl jasmonate (MJ), ethylene (ET), and brassinosteroids (BS). Several other components modulated by cold stress in various plants and their implication in stomatal function are listed in Table 12.2.

12.3 Signals That Could Convey Drought or Cold Stress to Stomata

Plants under drought or cold stress conditions respond by promoting the synthesis and mobilization of ABA in leaf vascular tissues. Under drought conditions, ABA translocation to the guard cells triggers stomatal closure (Seo and Koshiba 2011; Munemasa et al. 2015). ABA-induced stomatal closure is well established in several plant species, e.g., *Vicia faba*, *Commelina communis*, *Arabidopsis thaliana*, and *Pisum sativum*. Several other compounds also accumulate during drought, such as SA, proline, and PAs, all of which not only help in the acclimation of plant

Table 12.1 Multiple signals originating from different plant tissues during drought, which can induce stomatal closure

Signal	Reason for stomatal closure	References
Root		
Abscisic acid (ABA)	Induces stomatal closure through the activation of ROS and NO	Cummins et al. (1971)
Hydraulic pressure	Hydraulic signals trigger the production of ABA	Christmann et al. (2007)
Sulfate	Induces stomatal closure via QUAC1/ALMT12 anion channels	Malcheska et al. (2017)
Cytosolic pH	Acts prior to the ROS and NO	Wilkinson and Davies (1997) and Gonugunta et al. (2008)
1-Aminocyclopropane-1-carboxylic acid (ACC) (a ethylene precursor)	Ethylene induces H_2O_2 production via RBOHF	Desikan et al. (2006)
Leaves		
Salicylic acid (SA)	Induces superoxide and NO production	Mori et al. (2001)
Polyamines (PAs)	Induces stomatal closure by producing ROS and NO	Agurla et al. (2018)
Proline	Mechanism not known	Raghavendra and Reddy (1987)
Abscisic acid (ABA)	Induces stomatal closure through the activation of ROS and NO	Gonugunta et al. (2008)
Sphingosine kinase (SphK)	Elevates S1P and phyto-S1P	Guo et al. (2012)
Phospholipase C (PLC)	Promotes the production of phosphatidic acid; acts downstream of NO	Staxén et al. (1999)
Phospholipase D α/δ (PLDα and PLDδ)	Activates H_2O_2 and NO production	Jacob et al. (1999)
Inositol 1,4,5-trisphosphate (IP_3)	Releases internal stored calcium	Gilroy et al. (1990)
3′,5′-cyclic guanosine monophosphate (cGMP)	Mediates NO signaling by producing 8-nitro-cGMP	Joudoi et al. (2013)
Air		
High temperature	Decrease K^+ influx	Crawford et al. (2012)
Darkness	Induces the production of cytosolic pH and ROS by S1P and phyto-S1P	Ma et al. (2012)
High CO_2	Increases in ROS/NO and promotes Cl^- ion leakage from guard cells	Hanstein and Felle (2002) and Kolla et al. (2007)
Carbon monoxide (CO)	Induce the production of ROS and NO	She and Song (2008) and Song et al. (2008)
Ultraviolet-B (UV-B)	Induces stomatal closure through the production of H_2O_2 and NO	Nogués et al. (1999) and He et al. (2013)
Hydrogen sulfide (H_2S)	Acts downstream of NO during stomatal closure	Jing et al. (2012)
Hydrogen	Induces ROS and NO production	Xie et al. (2014)

Pressure, CO, and products of SphK/PLC/PLD and among the components listed above are known to promote stomatal closure. Further information on other components ABA, sulfate, cytosolic pH, H_2S, hydraulic pressure, CO, and products of SphK/PLC/PLD and UV-B are all listed in Gayatri et al. (2013), Agurla et al. (2014), Aliniaeifard and van Meeteren (2013), and Munemasa et al. (2015)

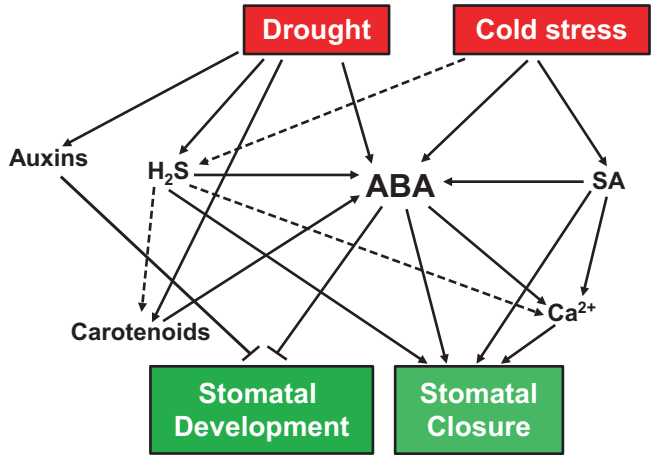

Fig. 12.2 Pathway of ABA accumulation under drought stress/cold during stomatal closure. Stress conditions (drought and cold) often lead to the production of ABA, SA, and auxins. Among these, ABA acts as central player by regulating stomatal closure. Under drought conditions, stomatal development is adversely affected, which leads to the decrease in stomatal frequency. Recent studies also suggest that drought also inhibits carotenoid synthesis, which further affects the synthesis of ABA. The stimulation of reactions with the experimental evidences is represented by solid arrows. The unknown interactions or the relationships where the evidences are lacking are represented by broken arrows. The inhibition/deficiency is indicated by ⊣

cells but also cause stomatal closure (Mori et al. 2001; Alcázar et al. 2010). A brief description of some of these components, known to induce stomatal closure, is given below.

12.3.1 Abscisic Acid

Among various plant hormones, abscisic acid (ABA) acts as an important regulator, capable of fine-tuning a spectrum of functions to enable plants to cope with different abiotic and biotic stresses (Finkelstein 2013). Plants under drought conditions respond by promoting the mobilization as well as the synthesis of ABA in leaf vascular tissues. Subsequently ABA is transported to the guard cells to trigger stomatal closure (Seo and Koshiba 2011; Munemasa et al. 2015). Recent investigations indicate that ABA further increases the levels of other compounds such as MJ, SA, and PAs (Alcázar et al. 2010). Detailed description of signaling events initiated by ABA in guard cells leading to stomatal closure is described in the following section.

In the signaling cascade of ABA-induced stomatal closure, the production of ROS (mainly H_2O_2) is an early event following which the nitric oxide (NO) is generated. As H_2O_2 generation is upstream to the NO, production of NO can also lead to the closing of stomatal aperture (Bright et al. 2006). Increase in the pH of the guard cell causing cytoplasmic alkalization is an early event in stomatal closure mediated by phytohormone abscisic acid (ABA). This alkalinity further mediates downstream signaling like production of secondary messenger, nitric oxide (Gonugunta et al. 2008). A phospholipid, phytosphingosine-1-P (phyto-S1P), produced by sphingosine kinase (SPHK) can activate phospholipase (PLD) and promote stomatal closure (Guo et al. 2012). During ABA induced closure of stomata, cytoplasmic alkalization is an early event, followed by the elevation of free calcium due to IP3, from the action of phospholipase C (PLC) (Staxen et al. 1999). The enzyme PLD is activated by ABA and one of the products of PLD, namely phosphatidic acid, can induce stomatal closure (Jacob et al. 1999)

Table 12.2 Components modulated by cold stress and their involvement in stomatal closure

Component	Effect of cold stress	References	Effect on stomata
Salicylic acid	Accumulation and improvement of cold tolerance	Miura and Tada (2014)	Closure
OST1 kinase	Regulation of CBF-dependent signaling for freezing tolerance	Ding et al. (2015)	Closure
ABA, brassinosteroids salicylic acid, and proline	Acclimation to improve cold tolerance	Kurbidaeva and Novokreshchenova (2011)	All these known to cause stomatal closure
Glycine-rich RNA-binding protein7 (GRP7)	Overexpression improves freezing tolerance	Kim et al. (2008)	Regulates opening/ closure
E. gunnii C-repeat binding factor (EguCBF1)	Overexpression helps in freezing tolerance	Navarro et al. (2011)	Stomatal density lowered
Heptahelical protein 1 (HPP1)	Deficiency causes hypersensitivity of plants to cold stress	Chen et al. (2010)	Mainly expressed in guard cells
Carotenoid deficiency	Inhibition of ABA and IAA biosynthesis and increased cold resistance	Du et al. (2013)	Deficiency caused wider stomatal aperture and faster wilting
Hydrogen sulfide (H_2S)	Alleviated cold stress by regulating MAPK signaling	Du et al. (2017)	Inhibition of stomatal opening
Heat shock protein26 (caHSP26)	Increases chilling stress tolerance by modulating the fluidity of the thylakoid membrane	Li et al. (2012)	Overexpression increases stomatal conductance
Oryza sativa cyclophilin 19-4 (OsCYP19-4)	Imparts freezing tolerance	Lee et al. (2016)	Protein targeted to guard cells
Calcium	Apoplastic Ca^{2+} increases on exposure to cold	Wilkinson et al. (2001)	Promotes stomatal closure
AtMYB4	Increase cold tolerance	Jung et al. (2008)	Enhanced stomatal closure
Overnight chill	Increases in guard cell sensitivity for CO_2	Allen et al. (2000)	Causes closure
Ca^{2+} ATPase (ACA8)	Imparts cold tolerance	Schiøtt and Palmgren (2005)	Expressed in guard cells needed for opening

Among the components listed above, ABA, SA, brassinosteroids, proline, OST1 kinase, free/external Ca^{2+}, and MYB4 are known to promote stomatal closure (Daszkowska-Golec and Szarejko 2013; Agurla et al. 2017; Agurla and Raghavendra 2016)

12.3.2 Methyl Jasmonate

Jasmonates, or jasmonic acid metabolites, play a key role in many biotic and abiotic stress responses. MJ induced stomatal closure in dose-dependent manner (Raghavendra and Reddy 1987) and was accompanied by the ROS production (Zhu et al. 2012a, b). While confirming the role of ROS, Suhita et al. (2004) suggested that alkalization of the cytoplasm is important and an early event during MJ-induced stomatal closure. The signaling components in response to MJ are similar to ABA guard cell signaling (Yin et al. 2016). Khokon et al. (2011a, b) reported that allyl isothiocyanate (AITC)-induced stomatal closure was also dependent on MJ-mediated ROS production.

Apart from ROS and NO, MJ is also capable of elevating other signaling components like Ca^{2+}. Further, there are reports demonstrating the role of cyclic adenosine 5′-diphosphoribose (cADPR) and cyclic guanosine 3′,5′-monophosphate (cGMP) in the elevation of Ca^{2+} during

MJ-induced stomatal closure (Hossain et al. 2014). Among the other regulators of MJ action are CPKs and mitogen-activated protein kinases (MPK9 and MPK12) (Khokon et al. 2015). ABA and MJ employ similar pathway for induction of ROS and NO and must interact with each other during stomatal closure (Munemasa et al. 2011).

12.3.3 Salicylic Acid and Its Esters

Phenolics are vital compounds involved in plethora of plant processes, including stress tolerance and disease resistance. Salicylic acid (SA) (2-hydroxy benzoic acid) is one of such phenolic compounds and plays a key role in processes such as systemic acquired resistance (SAR), fruit ripening, and even stomatal regulation (Dempsey et al. 2011; Miura and Tada 2014; Khan et al. 2015). The reports on the effect of SA on stomatal function have been conflicting. Rai et al. (1986) reported that stomata in *Commelina communis* open when treated with SA. Stomatal closure was observed in the presence of very low concentrations of SA (Hao et al. 2010; Khokon et al. 2011a, b). The other esters of SA such as acetylsalicylic acid (ASA), methyl salicylate (MeSA), and benzoyl salicylic acid regulate many developmental processes in plants (Kamatham et al. 2016; Klessig et al. 2016), but the effect of the esters on the modulation of stomatal function needs further studies.

Acetylsalicylic acid (ASA), an acetyl derivative of SA (commonly called aspirin) induced stomatal closure at high concentrations in the epidermal strips of *Commelina communis* (Larqué-Saavedra 1978, 1979). However, the role of signaling components such as ROS, NO, cytosolic pH, and calcium during ASA-induced stomatal closure is not clear. Similarly, MeSA is a volatile derivative of SA, occurring naturally in many plant species, and is considered as a mobile signal within plants, as it is transported to leaves to trigger the induction of SAR (Park et al. 2007; Dempsey and Klessig 2017). An important basis for SAR is through the production of reactive oxygen species (ROS); such rise in ROS can induce stomatal closure as well as plants' immunity response. However, no studies were conducted on the stomatal closure by MeSA (Dempsey and Klessig 2017). It is also important to study the interaction between the SA and its esters during the stomatal closure.

12.3.4 Phospholipids and Sphingolipids

Plants exposed to stress conditions often lead to the production of lipid signaling molecules from membrane lipids (Hou et al. 2016). Being major components of plasma membrane, phospholipids/sphingolipids have emerged as key signaling molecules in plants (Meijer and Munnik 2003; Testerink and Munnik 2005; Wang 2005). Phospholipids including phosphatidic acid (PA), phosphatidylinositol-4,5-bisphosphate (PIP2), phosphatidyl-inositol 3-phosphate (PI3P), phosphatidylinositol-1,4,5-trisphosphate (IP3), and diacylglycerol (DAG) regulate a wide range of developmental processes including stomatal closure (Choi et al. 2008; Kim et al. 2010; Misra et al. 2015). Phosphatidic acid (PA), the product of phospholipase C/D, induced stomatal closure by inhibiting K^{+}_{in} channel in the guard cells (Jacob et al. 1999). Further, it was observed that the levels of PA in *Vicia faba* guard cells increased on exposure to NO treatment. Treatment with inhibitors of either PLC or PLD inhibited the PA-induced stomatal closure, suggesting that NO may be involved in the production of PA and stomatal closure (Distéfano et al. 2008). The ABA-induced stomatal closure and production of NO were impaired in *pldα1* mutant guard cells (Distéfano et al. 2008, 2012). The *pldδ* mutants were impaired in stomatal closure by ABA, but the levels of NO and H_2O_2 still increased in response to ABA signaling. In contrast, *pldα1pldδ* double mutants were compromised in H_2O_2 and NO production in response to ABA. These results suggest that H_2O_2 and NO may be functioning downstream of PLDα/PLDδ and that PA was acting downstream of NO during closure (Uraji et al. 2012). Further, Zhang et al. (2004) and Bak

et al. (2013) discovered that PA could induce stomatal closure by inhibiting (ABI1) and activating NADPH oxidase.

Sphingolipid derivatives such as sphingosine-1-phosphate (S1P) and phytosphingosine-1-phosphate (phytoS1P) regulate multiple functions in plants besides stomatal closure (Coursol et al. 2005; Misra et al. 2015; Puli et al. 2016). Drought caused the production of S1P and increased the sensitivity to the ABA-mediated stomatal closure (Ng et al. 2001). ABA activates sphingosine kinases (SHPKs), which leads to the production of SIP. Current knowledge of further downstream signaling components of S1P is incomplete (Coursol et al. 2003).

12.3.5 Polyamines and Proline

Polyamines (PAs) are aliphatic low molecular weight, nitrogen-containing compounds involved in plant adaptation to stress conditions (Alcázar et al. 2010; Moschou and Roubelakis-Angelakis 2014; Pottosin et al. 2014). The accumulation of PAs during stress conditions such as drought (Alcázar et al. 2010) implies that PAs may have a role in stomatal function so as to conserve water loss. PAs are oxidized by amine oxidases like copper amine oxidase (CuAO)/diamine oxidase (DAO) and polyamine oxidase (PAO), releasing H_2O_2 which in turn could act as signal in many physiological processes, including stomatal closure (Cona et al. 2006; Alcázar et al. 2010; Moschou and Roubelakis-Angelakis 2014). The oxidation of PAs through CuAO and PAO is an important source of ROS. Liu et al. (2000) suggested that PAs induced stomatal closure by ROS production, inhibiting the inward K^+ currents in *Vicia faba*. Later, An et al. (2008) discovered that ABA-induced ROS production was mediated through the copper amine oxidase (CuAO) during stomatal closure. Agurla et al. (2018) reported that, PAs induced production of NO as well as ROS in stomatal guard cells of *Arabidopsis thaliana* and suggested that both NADPH oxidase and amine oxidases were responsible for ROS or NO production during PA-induced stomatal closure. It is quite reasonable to expect that H_2O_2 a product of PA oxidation can elevate NO. Further work is required to understand if polyamines have a direct or indirect effect on the production of NO and ROS in stomatal guard cells.

Proline is a nitrogenous, osmolyte known to accumulate under abiotic stresses and help in stress tolerance (Albert et al. 2012). There is a correlation between drought stress and proline content in plants. Proline can regulate stress by acting as a signaling component as well as antioxidant. The mechanism of proline signaling during drought is not clear. Liu et al. (2013) reported that overexpression of a ring-finger protein from *Zea maize* (ZmRFP) showed better drought resistance by decreasing stomatal pores. In addition to this, exogenous application of proline induced partial stomatal closure (Raghavendra and Reddy 1987; Hayat et al. 2012). The available reports suggest that proline could act as an osmolyte under drought stress, but the mechanism of action in relation to stomatal closure needs further studies.

12.4 Mechanism of Stomatal Closure by ABA: A Case Study

ABA-induced stomatal closure is well established in several plant species, e.g., *Vicia faba*, *Commulia communi*, *Arabidopsis thaliana*, and *Pisum sativum*. The presence of ABA triggers the efflux of anions and potassium via plasma membrane ion channels, resulting in decrease of turgor pressure in guard cells and stomatal closure. ABA-induced stomatal closure is mediated by many signaling components like cytoplasmic pH, reactive oxygen species (ROS), reactive nitrogen species (NO), cytosolic Ca^{2+}, G-proteins, protein kinases as CDPK and MAPK, protein phosphatases, phospholipases, and sphingolipids (Raghavendra et al. 2010; Gayatri et al. 2013; Song et al. 2014; Laxalt et al. 2016; Agurla et al. 2017). Various secondary messenger molecules

like ROS, NO, cytosolic Ca^{2+}, and protein kinases (OST1 kinases) and protein phosphatases (ABI1 and AB12) form the major ABA core signaling network to induce stomatal closure (Lee and Luan 2012; Gayatri et al. 2013; Raghavendra et al. 2010). However, the mechanism of transportation of ABA into the stomatal guard cells is not clearly understood.

ABA-binding proteins of *Arabidopsis* have recently been identified by two research groups (Park et al. 2009; Ma et al. 2009). ABA binding to the receptor (RCAR/PYR1/PYL proteins) causes conformational changes in the core receptor complex, in such a way that it facilitates the reversible binding of 2C type protein phosphatases such as ABI1 and ABI2 (Raghavendra et al. 2010). The binding of type2C protein phosphatases releases the inhibitory effect of PP2C on SNF1 (sucrose non-fermenting kinase1) protein kinases leading to the activation of NADPH oxidase and release of ROS (Fig. 12.3) (Fujita et al. 2009). In ABA-mediated signaling, increase in pH is also recorded (MacRobbie 1998). The kinetic studies show that the cytosolic alkalization precedes the ROS production in guard cells (Suhita et al. 2004; Gonugunta et al. 2008). The interactions between cytosolic pH and ROS need to be examined further. In addition to ROS, NO, and pH, cytosolic free calcium also acts as a vital secondary messenger during ABA-induced stomatal closure. ABA elevates cytoplasmic calcium and helps in the activation of S-type anion channels (Munemasa et al. 2015). Further, calcium-dependent protein kinases (CDPKs) are known to activate slow anion channel SLAC, like in the case of OST1. Along with OST1 kinase and CDPKs, another intricate part of the ABA signaling consists of mitogen-activated protein kinases (MAPKs) which act downstream of H_2O_2 (de Zelicourt et al. 2016). As a result of the above signaling events, key ion channels located in the plasma membrane are activated by ABA. These include S-type anion channels and outward K^- channels, whereas inward K^+ channels are inactivated, leading to decrease in ionic status in guard cells (Kim et al. 2010; Lee and Luan 2012). A comprehensive list of signaling components modulated by ABA is listed in Table 12.3.

12.5 Convergence of Signals During Stomatal Closure by Different Factors

Multiple signaling components in guard cells lead to stomatal closure. Upon exposure to the abiotic or biotic stress, guard cells initiate signal transduction process. These signals from the environment are sensed and integrated by the guard cells so as to adapt quickly to stress conditions. Though our knowledge on the effect of several signaling components on stomatal closure is improving, the interactions among them are not completely understood. Both plant hormones (such as ABA or MJ) and microbial elicitors induce stomatal closure with the help of signaling components/secondary messengers such as ROS, NO, cytosolic pH, calcium, and anion channels in guard cells (Agurla et al. 2017). These different signaling events ultimately converge to trigger the loss of ions and subsequently the turgor in guard cells, thus causing stomatal closure.

Among the signaling components, ROS, NO, and Ca^{2+} are identified as a key converging points. Detailed reviews are available on the role of ROS, NO, and calcium during stomatal closure (Kim et al. 2010; Gayatri et al. 2013; Kollist et al. 2014; Murata et al. 2015). The increase in the levels of ROS, NO, or cytosolic free Ca^{2+} in stomatal guard cells trigger multiple events either downstream or upstream (Agurla and Raghavendra 2016). The rise in ROS of guard cells initiates downstream effects such as increase in the levels of NO, Ca^{2+}, and cytosolic pH (Wang and Song 2008; Song et al. 2014). The upstream action of ROS to NO was confirmed in several reports (Bright et al. 2006; Gonugunta et al. 2008; Gayatri et al. 2017). Strong interactions between ROS, NO, and pH appear to be possible but need further study. The cytosolic pH, G-proteins, and MAP

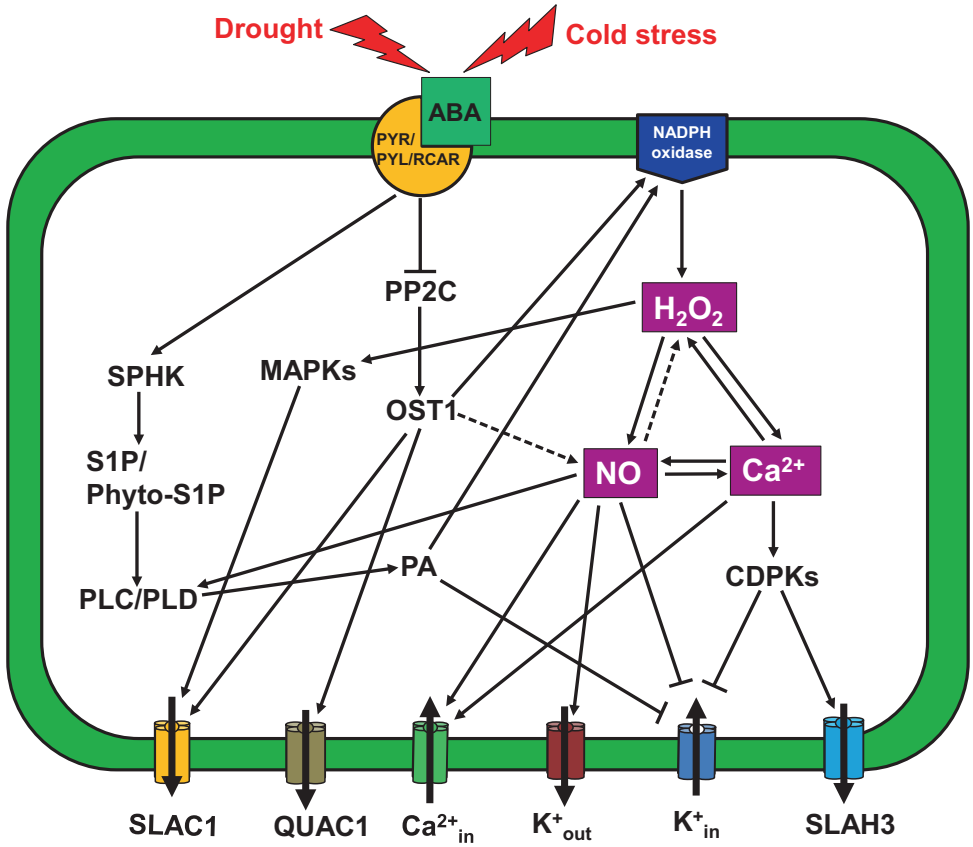

Fig. 12.3 A scheme of signal transduction mechanism under abiotic (drought/cold) stress was mediated by several secondary messengers in guard cells during stomatal closure. Abiotic stresses like drought and cold stress induce the production of plant hormones such as ABA, SA, MJ, and osmoregulatory compounds like proline and PAs. Among these, the mechanism of ABA-induced stomatal closure was studied in detail. ABA upon binding to receptor on the plasma membrane such as PYL/PYR/RCAR abolishes the inhibitory effect of PP2C phosphatases (ABI1 and ABI2) on sucrose non-fermenting (SNF)-related kinase such as OST1. This leads to activation of NADPH oxidase and elevation of ROS/NO production. ROS further elevates the levels of NO and Ca^{2+} (available literature clearly indicates the production of ROS, NO, and Ca^{2+}in stomatal guard cells during abiotic stresses, but the mechanism of interaction between major signaling components such as ROS, NO and Ca^{2+} is not clear). Elevated levels of ROS/NO/Ca^{2+} activate different anion channels like SLAC1/SLAH3/QUAC1; simultaneous inhibition of K^+ ion accumulation in the guard cells was accomplished by the inhibition of K^+_{in} channels in the plasma membrane. Apart from this, ABA also activates the production of lipids like PA, S1P, and phyto-S1P. PA activates the ROS production and parallelly inhibits the activity of K^+_{in} channels. All these events lead to the removal of prevailed osmoticum in the guard cells and induce stomatal closure. The stimulation of reactions with the experimental evidences is represented by solid arrows. The expected interactions or the relationships where the evidence is not clear are indicated by broken arrows. The inhibition/deficiency is indicated by ⊣

kinases may be acting in parallel during stomatal closure. Detailed description of the convergence and divergence of signaling components can be found by the reader in the review by Agurla and Raghavendra (2016).

12.6 Concluding Remarks

Stomatal movements are due to the unique operation of signaling components in guard cells, common during closure by ABA or elicitors. These

Table 12.3 Signaling components in guard cells involved in stomatal closure by ABA

Signaling component	Mechanism of action	References
Reactive oxygen species (ROS)	Elevates NO and Ca^{2+} and other downstream events	Kwak et al. (2003)
Nitric oxide (NO)	Modulates the activity of PLDδ and ion channels	Desikan et al. (2002)
Cytosolic pH	Acts upstream of ROS, NO, and Ca^{2+} during stomatal closure	Irving et al. (1992)
Cytosolic free Ca^{2+}	Activates anion channels and inhibits the K^{+}_{in} channels, all leading to stomatal closure	Schroeder and Hagiwara (1990)
3′-Phosphoadenosine 5′- phosphate	Acts as a secondary messenger and regulates the activity of ROS and Ca^{2+}	Pornsiriwong et al. (2017)
OST1 kinase	Regulates the activity of RBOHF, NO, and SLAC/QUAC-type anion channels	Acharya et al. (2013) and Wang et al. (2015)
Reactive carbonyl species (RCS)	Acts downstream of H_2O_2	Islam et al. (2016)
Phytosphingosine-1-phosphate and sphingosine-1-phosphate	Elevates ROS, cytosolic pH, and NO	Ma et al. (2012) and Puli et al. (2016)
PYR/PYL and RCAR	Function as ABA receptors during stomatal closure	Park et al. (2009) and Ma et al. (2009)
PYR1 interacting partner (HAB1)	Functions as a negative regulator of ABA signaling	Nishimura et al. (2010)
Protein phosphatases type 2C (ABI1 and ABI2)	Act as negative regulators of ABA signaling	Merlot et al. (2001)
ABA-activated protein kinase (AAPK)	Mutation of AAPK leads to impairment in ABA guard cell signaling	Li et al. (2000)
Calcium-dependent protein kinases (CPK3 and CPK6)	Modulate ion channel activity in response to ABA	Mori et al. (2006)
Myosin-activated protein kinases (MAPK9 and MPK12)	Function downstream of ROS to regulate guard cell ABA signaling	Jammes et al. (2009)
H^{+} proton pump (ATPase)	Causes hyperpolarization of plasma membrane, activation of K^{+} influx	Goh et al. (1996)
Phosphatidic acid (PA)	Activates NADPH oxidase and inhibits K^{+}_{in} channels	Zhang et al. (2004)
Phosphatidylinositol-3-phosphate-5-kinases (PI3P5K)	Mediates vacuolar acidification during ABA-mediated stomatal closure	Bak et al. (2013)
Guard cell hydrogen peroxide resistance 1 (GHR1)	Activation of S-type anion channels during ABA-induced stomatal closure; depends on ABI2 but not on ABI1	Hua et al. (2012)
Calcineurin B-like proteins (CBL1, CBL9 and CIPK 29)	Phosphorylates RBOHF and enhances ROS production	Drerup et al. (2013)
Sphingosine kinase (SphK)	Increases S1P and Phyto-S1P	Guo et al. (2012)
Phospholipase C (PLC)	Produces phosphatidic acid, acts downstream of NO	Staxén et al. (1999)
Phospholipase D α/δ (PLDα and PLDδ)	Involved in the production of H_2O_2 and NO	Jacob et al. (1999)
Inositol 1,4,5-trisphosphate (IP_3)	Promotes calcium release from internal stores	Gilroy et al. (1990)
3′,5′-Cyclic guanosine monophosphate (cGMP)	Mediates NO signaling by producing 8-nitro-cGMP	Joudoi et al. (2013)
Cyclic ADP ribose (cADPR)	Mobilizes calcium and induces reduction in turgor pressure of guard cells	Leckie et al. (1998)
ROP2 GTPase (ROP2)	Acts as a negative regulator of ABA signaling	Hwang et al. (2011)
G-protein α-subunit (GPA1)	Regulates ROS/NO production in response to ABA	Coursol et al. (2003)

(continued)

Table 12.3 (continued)

Signaling component	Mechanism of action	References
Coronatine insensitive 1 (COI1)	Receptor for MJ during stomatal closure	Munemasa et al. (2007)
Sulfate	Induces stomatal closure via QUAC1/ALMT12 anion channels	Malcheska et al. (2017)
Calcium-dependent protein kinases 10 (CDPK10)	Interact with HSP1 during stomatal closure by ABA and Ca^{2+}	Zou et al. (2010, 2015)
Pyrabactin resistance-like protein 8 (PYL8)	Acts as a receptor for ABA	Lim et al. (2013)

Further information on above components and others involved in stomatal closure in response to other hormones/microbial elicitors can be found in reviews of Lee and Luan (2012), Sawinski et al. (2013), Agurla et al. (2017), Zhang et al. (2014), and Kollist et al. (2014)

components play an important role not only in stomatal closure but also in integrating stimuli from abiotic or biotic stress. The patterns and action sequence of signaling components during stomatal closure have mostly been worked out in detail with ABA. For example, cytoplasmic pH acts as an early signaling component, followed by the production of ROS and NO, all leading to a rise in Ca^{2+} and modulation of ion channels to trigger efflux of K^+/anions. During drought or cold stress, there is often decrease in carbon assimilation capacity. The resulting increase in intercellular CO_2 can also cause stomatal closure. Such cross talk between H_2O and CO_2 signaling is essential in keeping up the balance between the restriction of water loss and sustenance of CO_2 uptake.

Among other aspects which need to be examined further are the possible sensors for CO_2, humidity, turgor, or others. Similarly, the extent of bound and free ABA in leaves or roots and its consequences in apoplastic pH changes is quite crucial for modulating stomatal closure and contribution to the plant tolerance to drought/cold stress. The mechanisms of modulation of hydraulic signals from roots and other transmissions to root are relevant but not understood. There are additional topics that would improve our understanding of stomatal function and its relevance to plant adaptation to abiotic or biotic stress. Such topics include the modeling/system biology approach, evolutionary trends in stomatal function, sensing of humidity, and stomatal development itself (Aliniaeifard and van Meeteren 2013; Chater et al. 2013, 2014; García-Mata and Lamattina 2013; Roychoudhury et al. 2013; Lawson and Blatt 2014; Medeiros et al. 2015).

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13

Mechanisms of Maturation and Germination in Crop Seeds Exposed to Environmental Stresses with a Focus on Nutrients, Water Status, and Reactive Oxygen Species

Yushi Ishibashi, Takashi Yuasa, and Mari Iwaya-Inoue

Abstract

Environmental stresses can reduce crop yield and quality considerably. Plants protect cell metabolism in response to abiotic stresses at all stages of their life cycle, including seed production. As the production of vigorous seeds is important to both yield and crop growth, we analyzed causes of yield loss and reduced grain quality in staple crops exposed to environmental stresses such as drought and temperature extremes, with a focus on the remobilization of nutrients and water status during seed filling. Because water is one of the factors that limit seed development, seeds must have mechanisms that allow them to withstand water loss during seed maturation. In addition, analysis of the effects of reactive oxygen species (ROS) on transcription regulation and signaling should help to elucidate the regulation of seed dormancy and germination. In this review, we focus on nutrient remobilization, water mobility, plant hormones (gibberellins, abscisic acid, and ethylene), and ROS in sink and source organs and describe how rice, wheat, barley, soybean, and cowpea plants control seed maturation and germination under environmental stresses.

Keywords

Seed-filling stage · Remobilization of nutrient · Dormancy · Germination · ROS · Oxidative window · ABA · GA · Physical states of water · Environmental stress · Preharvest sprouting · Seed quality · Cowpea (*Vigna unguiculata*) · Soybean (*Glycine max*) · Rice (*Oryza sativa*) · Wheat (*Triticum aestivum*) · Barley (*Hordeum vulgare*)

Abbreviations

ABA	Abscisic acid
AQP	Aquaporin
CAT	Catalase
CmACS1	1-Aminocyclopropane-1-carboxylate synthase

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DAF	Days after flowering
GAMyb	GA Myb transcription factor
GAs	Gibberellins
GSS	Green stem syndrome
H_2O_2	Hydrogen peroxide
LEA	Late embryogenesis abundant
MRI	NMR imaging
NMR	Nuclear magnetic resonance
PHS	Preharvest sprouting
PIP	Plasma membrane intrinsic protein
PK	Protein kinase
PKABA	ABA-responsive protein kinase
ROS	Reactive oxygen species
SUT1	Sucrose transporter
T_1	NMR spin-lattice relaxation time
T_2	NMR spin-spin relaxation time
TIP	Tonoplast intrinsic protein

13.1 Seed Development and Maturation Under Environmental Stresses

Abiotic stresses associated with water, such as drought, salinity, continual rain, and temperature extremes, reduce plant growth and, as a result, decrease yield and grain quality (FAO 2017). The production of highly vigorous seeds is important to a stable yield (Hilhorst et al. 2010; Finch-Savage and Bassel 2016). Plants protect cell metabolism in response to drought stress as abiotic stress and an endogenous condition as a maturing seed during seed desiccation (Walters et al. 2005; Devic and Roscoe 2016). Orthodox seeds lose water to around 10% of fresh weight and can survive for a long time while being able to respond to pathogens, light, and auxin (Righetti et al. 2015). Desiccation-tolerant seeds occurred in maturation and dormancy is controlled by abscisic acid (ABA) signaling (Bewley et al. 2013; Zinsmeister et al. 2016; Leprince et al. 2017). The transcription of ABA catabolism genes *CYP707A1* and *CYP707A3* is downregulated and that of ABA biosynthesis genes, including *NCED*, is upregulated by ABI4 and other regulators, resulting in ABA accumulation to initiate dormancy (Fang and Chu 2008). Other key dormancy-controlling genes, including *ABI3*, *ABI4*, *DOG1*, *DEP*, and *SPT*, are activated during seed maturation to induce and maintain primary seed dormancy (Nambara et al. 2010; Shu et al. 2016).

Late embryogenesis abundant (LEA) proteins, a diverse family, are accumulated during seed desiccation to protect seeds against dehydration (Tunnacliffe et al. 2010; Battaglia and Covarrubias 2013; Delahaie et al. 2013; Ling et al. 2017). Dehydrins are major LEA proteins associated with water status in cells. Besides, cellular viscosity increases dramatically, and the cytoplasm transforms into a "glassy state" during seed drying (Buitink and Leprince 2004, 2008; Walters et al. 2005). The sugar as glassy states apparently trap macromolecules in a stable medium, hence preventing cell deterioration (Hoekstra et al. 2001). Trehalose protects proteins from denaturation induced by desiccation, likely by replacing water molecules at their surface (Crowe et al. 1992; Sakurai et al. 2008). Molecular modeling has indicated that as the water content approached 0.1 gH_2O/gdw, the matrix formed a large interconnected trehalose skeleton with a minimal number of bound water molecules scattered in the bulk (Weng et al. 2016). Group 3 LEA proteins interact with trehalose and form a tight glassy matrix in the dry state (Iturriaga 2008). Additionally, the proteins and sugars such as raffinose family oligosaccharides induced during seed maturation protect the membranes and biomolecules in the embryo (Gangola et al. 2016).

Although a number of molecular and cellular processes in maturing seeds of *Arabidopsis* have been reported in detail (To et al. 2006; Santos-Mendoza et al. 2008; Manfre et al. 2009), the problem of the reduction of yield and grain quality in staple crops under environmental stresses has not been solved. Seed development and morphology in crop seeds are notably different from those in *Arabidopsis*. In monocots, nutrients accumulate mainly in the starchy endosperm (Fig. 13.5a–c), whereas in dicots nutrients such as starch, protein, and lipid generally accumulate in massive embryos that consist of thick cotyledons

as a main food store, with hypocotyls, radicles, and plumules surrounded by trace proportion of endosperm layers (Fig. 13.5) (Bewley et al. 2013).

Here, we analyze serious agricultural problems that result in yield loss and lower grain quality in crops, such as cowpea, soybean, rice, and wheat exposed to temperature and water stresses, with particular focus on the remobilization of nutrients and physical states of water through GA/ABA metabolism during seed filling.

13.2 Remobilization of Nutrients and Water in Sink and Source Organs of Legume Crops Under Drought Stress

13.2.1 Homeostatic Strategy at the Reproductive Stage in Cowpea, a Drought-Tolerant Legume Crop

Global production of cowpea (*Vigna unguiculata*) seeds in 2010 was 5.5 million tons; African countries were responsible for 94% of this production (CGIAR 2017). Cowpeas are widely cultivated in the semiarid Sahelian area, which has 300–800 mm of rainfall per year with high evaporation, where many other crops cannot grow (van Duivenbooden et al. 2002; Hall 2004). Cowpea grains mainly contain about 55.5% carbohydrate, 23.9% protein, and 15.5% water (MEXT 2017, Fig. 13.1) and are the most important source of protein and starch of the diet in West and Central Africa (Ogbonnaya et al. 2003; Adebooye and Singh 2008). Drought stress suppresses photosynthesis in cowpea because of stomatal closure at both the vegetative stage (Osonubi 1985; Imamura et al. 2010) and the reproductive stage (Egashira et al. 2016). Although approximately 64% of carbon accumulation in cowpea seeds depends on photosynthates produced in leaves at grain filling (Pate et al. 1983), grain dry weight does not differ significantly between unstressed and drought-stressed cowpeas (Abayomi et al. 2000; Muchero et al. 2008, Egashira et al. 2016). Although the mechanism of drought tolerance of cowpea has been investigated at the molecular level at the vegetative stage (Iuchi et al. 2000), there are very few studies of the grain-filling stage at which cowpeas suffer most frequently from drought stress (Citadin et al. 2011).

Drought stress drastically accelerates leaf senescence and seed maturation compare to the control at the grain-filling stage (Fig. 13.1b, c). In cowpeas under water deficit, the expression of genes related to carbohydrate synthesis, degradation, and transport drastically changes in sink and source organs. Marked increases in both α- and β-amylase activities were observed in the leaves of cowpea subjected to drought, and thus the starch content of these leaves was significantly lower than that of the controls (Egashira et al. 2017). Photoassimilates stored in the flower stalk, petiole, stem, hypocotyl, and root as temporary sink and source organs are rapidly degraded and translocated to seeds. Consequently, cowpea grain yield is maintained, despite a dramatic decrease in the photosynthetic rate, by the translocation of photoassimilates from leaves via the temporary sink and source organs, especially from flower stalks to seeds.

Nitrogen remobilization was also observed from leaves through flower stalks to seeds under drought stress (unpublished data); the elongation of the flower stalk allows it to function as a sink organ that buffers the carbohydrate and nitrogen pools translocated from senescing leaves to growing seeds under drought stress (Fig. 13.1). Drought stress in cowpea at the seed-filling stage may promote asparagine biosynthesis and proline degradation by reducing photoassimilate concentrations in leaves (Goufo et al. 2017). The expression of amino acid catabolism-related genes, such as those for proline dehydrogenase (*VuProDH*), asparagine synthase (*VuASN1*), and branched-chain amino acid transaminase (*VuBCAT2*), was also upregulated in cowpea seedlings under sucrose starvation (Kaneko et al. 2013). It means that drought stress in cowpea accelerates nutrient translocation from senescing leaves to developing seeds, but does not increase proline biosynthesis for maintaining leaf drought tolerance at the vegetative stage. On the basis of these results, it is suggested that developing seeds

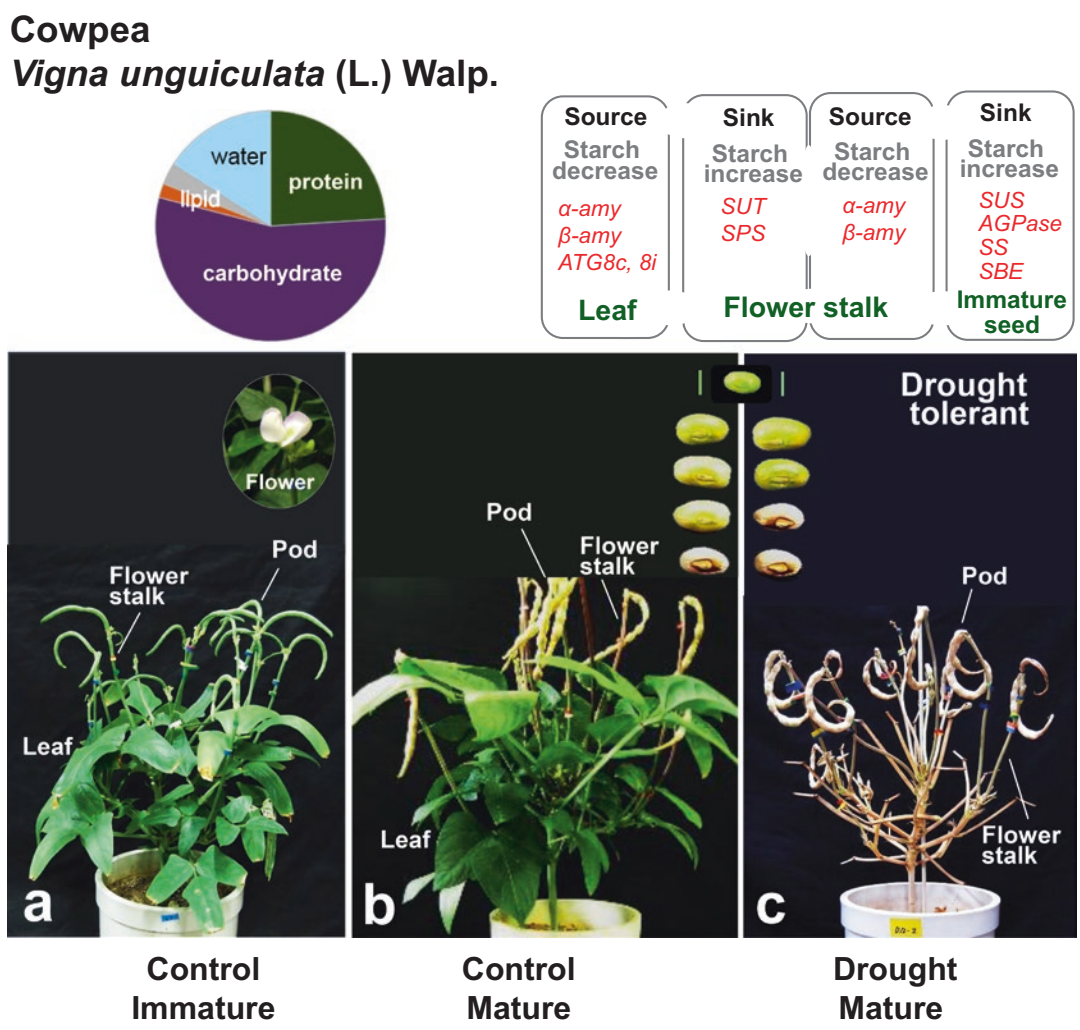

Fig. 13.1 Effect of drought treatment on cowpea and the expression of starch synthesis- and degradation-related genes, and sucrose transporter genes in seeds, leaves, and flower stalks at the grain-filling stage. (**a**) A cowpea plant at seed developing stage under control conditions. (**b**) A plant and developing seed 0, 3, 7, 10, and 14 days after irrigation as control conditions. (**c**) A plant and developing seed 0, 3, 7, 10, and 14 days after the onset of drought treatment under drought stress. Red letters indicate upregulated genes induced by drought stress at 9 days after treatment. Cowpea plants are of "IT-99K-241-2," a drought-tolerant line bred in IITA, Nigeria. *SUS* sucrose synthase, *AGPase* ADP-glucose pyrophosphorylase, *SS* starch synthase, *SBE* starch-branching enzyme, *SUT* sucrose transporter, *SPS* sucrose-phosphate synthase, *α-amy* α-amylase, *β-amy* β-amylase. Scale bar of seeds indicates 5 mm. Based on information in Egashira et al. (2017), mature seed content in a circle graph is indicated by ratio of protein (green), carbohydrate (violet), lipid (orange), mineral and others (gray), and water (blue). The data are based on "Standard tables of food composition in Japan, #04017" (MEXT 2017). (Photograph courtesy of Yuya Hashiguchi and Takashi Yamauchi, Kyushu University)

accelerate the uptake of carbohydrates from source organs, leading to severe low-energy stress in leaf tissues. Additionally, we have shown that sucrose starvation stress enhances the expression of *VuATG8i*, *VuATG8c*, and *VuATG4* coding autophagy-related (ATG) protein family in cowpea seedlings (Kaneko et al. 2013). Drought stress also induced elevation in gene expression of *VuATG8i* and *VuATG8c* in cowpea leaves. Atg8, in its lipidated form, is localized to the

isolation membrane and the autophagosome (Kirisako et al. 1999; Nakatogawa et al. 2007). Autophagy, known as a nutrient recycling system, plays a crucial role in nitrogen and starch remobilization to seeds during leaf senescence (Liu and Bassham 2012; Pottier et al. 2014; Ren et al. 2014).

Drought stress in cowpea did not affect the germination rate, indicating that seed viability and grain quantity are maintained (Egashira et al. 2016). Aquaporins (AQPs) are membrane channels that finely control the passage of water through membranes as regulatory mechanisms for tonoplast intrinsic proteins (TIPs) and plasma membrane intrinsic proteins (PIPs) in plants (Maurel et al. 2015; Chaumont and Tyerman 2017). Arabidopsis TIP1;1 (γ-TIP) is a member of the tonoplast family of AQPs (Ma et al. 2004). In the mature seed coat of pea (*Pisum sativum* L.), strong hybridization signals were observed with the probe for *PsPIP1-1*, but the transcripts of *PsPIP2-1* and *PsTIP1-1* were undetectable (Schuurmans et al. 2003). Functional characterization of PsPIP2-1 and PsTIP1-1 heterologously expressed in *Xenopus* oocytes showed that these proteins are AQPs. The expression of seed-specific AQPs is thought to be crucial during seed ripening (Takahashi et al. 2004; Gattolin et al. 2011). In cowpea seeds, the level of γ-TIP increased with ripening; γ-TIP appears earlier under drought stress, and its level was maintained until 14 days after the onset of treatment. In particular, γ-TIP was expressed in the hypocotyls, radicles, and plumules, but not in the cotyledons. The TIP proteins increase transcellular water flow by increasing the effective cross-section of the cytoplasm and facilitate osmotic adjustment between the cytoplasm and the vacuole (Mao and Sun 2015). Earlier expression of γ-TIP in cowpea seeds, especially hypocotyls, radicles, and plumules, exposed to drought stress suggests a survival strategy (Egashira et al. 2016). In another legume, broad bean (*Vicia faba*), the level of α-TIP AQP distribution in seeds markedly increases with advanced maturation and is maintained during dry seed storage (Béré et al. 2017). These results indicate that AQPs contribute to maintaining the seed integrity of the broad bean. Understanding the mechanisms of nutrient and water remobilization in cowpea will contribute to breeding new crops that can be grown under drought conditions.

13.2.2 Changes in the Storage Components in Soybean Caused by Drought Stress Accompanied by Sink-Source Imbalance

Soybean (*Glycine max*) is the most widely grown legume crop, with a total production of 349.8 million tons in 2016/2017 (FAO 2017). Soybean seeds are an exceptional source of essential nutrients and mostly contain 33.8% protein, 29.5% carbohydrates, 19.7% total fat, and 12.4% water (MEXT 2017, Fig. 13.2). Under drought stress, the productivity of soybean is severely limited because the seed size, number, and yield are all reduced compared to those of cowpea as described above (Dornbos and Mullen 1992; Frederick et al. 2001; Brevedan and Egli 2003; Sinclair et al. 2009; Egashira et al. 2016). In soybean, yield loss is most severe when drought stress is applied throughout the seed development period (reproductive stages R5-R7), resulting in a reduction of 45% in R5 and 88% in R7 (Eck et al. 1987). During these stages, the pods start to set, and the seed composition rapidly increases with pod elongation. The effects of drought on the expression of protein and lipid biosynthesis and degradation-related genes in soybean seeds treated at R5 are shown in Fig. 13.2. Seed maturation is accompanied by a profound reorganization of numerous protein storage vacuoles (Bewley et al. 2013). The expression of the protein storage-related genes *GmGy4*, *Gmβ-conglycinin*, and protein folding gene, *GmCyp1* decreases with drought stress. In contrast, the expression of *GmCys-proteinase*, a key enzyme participating in protein degradation, is elevated. In legumes, cysteine proteinases are involved in the catabolism of seed protein reserves (Becker et al. 1994; Tiedemann et al. 2001), and the proteinase activity is closely related to environmental stress conditions, for example, dehydration

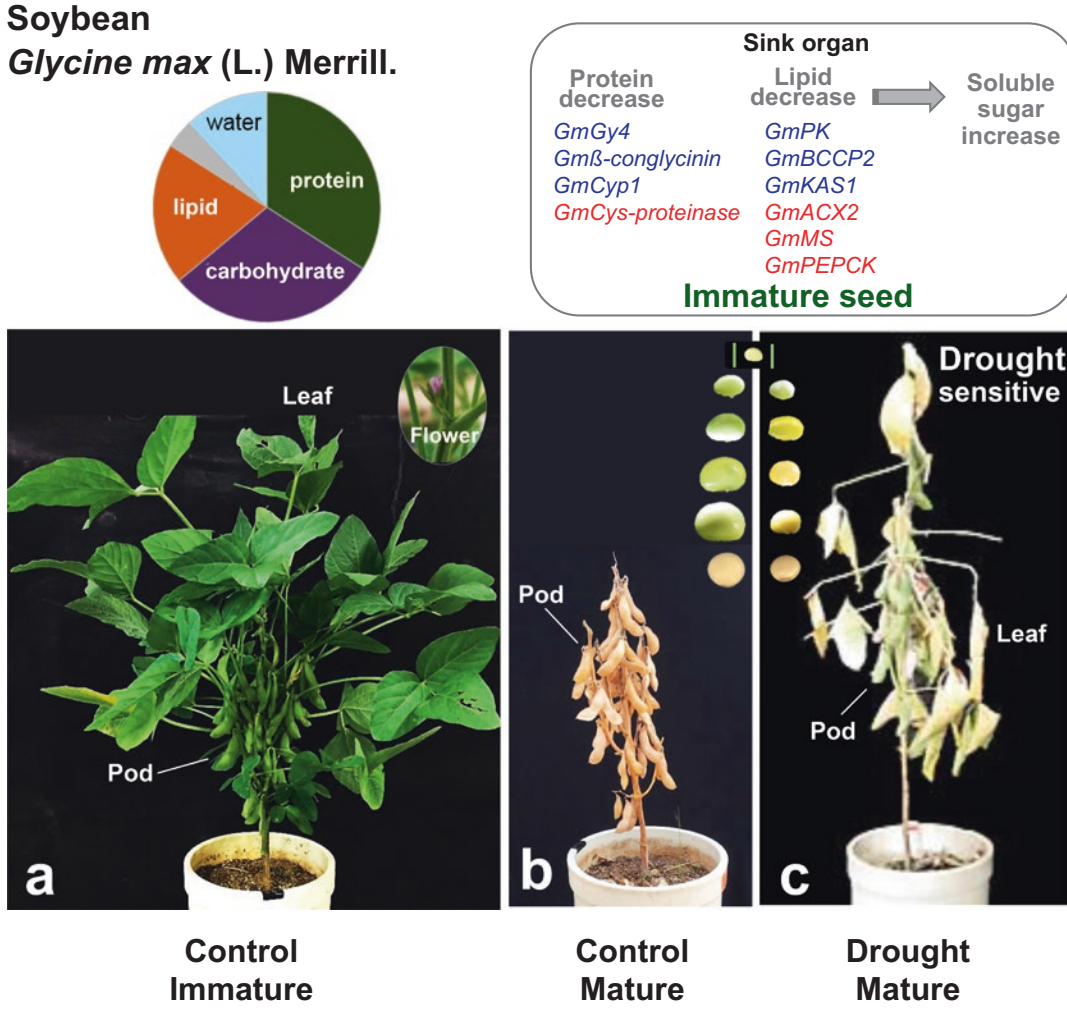

Fig. 13.2 Effect of drought stress on soybean and the expression of protein and lipid synthesis-related genes and lipid degradation-related genes in seeds at the grain filling stage (**a**) A soybean plant at seed developing stage at R 5.5 under control conditions. (**b**) A soybean plant and developing seed 0, 10, 19, 24, and 29 days from R5 and mature seed at harvest stage under control condition. (**c**) A plant and developing seed 0, 10, 19, 24, and 29 days after the onset of drought treatment at the R5 stage and mature seed at harvest stage under drought stress. Soybean plants are of "Fukuyutaka," a popular cultivar in Japan. Red letters indicate upregulated genes, while blue letters indicate downregulated ones induced by drought stress at 29 days after treatment. *Gm* glycine max, *Gy* glycinin gene, *Cyp* cytochromes P450, *Cys* proteinase *cysteine proteases*, *PK* pyruvate kinase, *BCCP* biotin carboxyl carrier protein, *KAS* ketoacyl-ACP synthase, *ACX* acyl-CoA oxidase, *MS* malate synthase, *PEPCK* phosphoenolpyruvate carboxykinase, *ATG* autophagy, *Carbo.* carbohydrate. Scale bar of seeds indicates 5 mm. Based on information in Nakagawa et al. (2017), mature seed content shown in a circle graph is based on "Standard tables of food composition in Japan, #04023" (MEXT 2017). (Photograph courtesy of Andressa C. S. Nakagawa, Yuki Tomita and Daichi Tajima, Kyushu University)

and salt stress (Koizumi et al. 1993; Jones and Mullet 1995). In addition, the decrease in protein content is regulated in a symbiotic interaction between soybeans and rhizobia under drought stress (Kunert et al. 2016).

Drought stress also alters the fatty acid composition of soybean seeds and affects total oil levels and the stability of oil, especially during the seed-filling stage (Bellaloui et al. 2013). In soybean seeds exposed to drought stress, the

expression of key genes in lipid biosynthesis (*GmPK*, *GmBCCP2*, and *GmKAS1*) is reduced (Fig. 13.2c). The disruption of the β_1-subunit of protein kinase (PK) caused a 60% reduction in *Arabidopsis thaliana* seed oil content (Andre et al. 2007). Additionally, ketoacyl-ACP synthase carboxylates malonyl-ACP from acetyl-coenzyme A, whereas biotin carboxyl carrier protein (BCCP) facilitates the transfer of carboxyl groups to acetyl-CoA (Thelen and Ohlrogge 2002; Baud et al. 2008). A 38% reduction in BCCP2 protein content by antisense expression reduced the oil content in developing *Arabidopsis* seeds by 9% compared to the wild type (Thelen and Ohlrogge 2002). The expression of key genes in lipid degradation (*GmACX2*, *GmMS*, and *GmPEPCK*) is increased by drought stress (Nakagawa et al. 2018).

Soluble sugar content in soybean seeds is significantly increased when lipid content is reduced by drought (Nakagawa et al. 2018). Overall, the above data indicate that the content of lipids in ripening seeds under drought stress is reduced because of the suppression of their biosynthesis and promotion of their degradation and sugars consequently accumulate in the seed. The accumulated sugars may be used for stabilizing membranes, liposomes, and proteins followed by maintaining essential growth and metabolism of seed maturation accompanying with water loss (Crowe et al. 1992; Gibson 2005; Buitink et al. 2006).

Unlike the leaves of other annual legume crops such as cowpea (Fig. 13.1b), soybean leaves drop, and the stems lose their green color with seed maturation (Fig. 13.2b). Soybean flowers continue to open for a long time, but the pods mature simultaneously (Zheng et al. 2003). A soybean-specific phenomenon called green stem syndrome (GSS) prevents soybean stems and leaves from drying down properly, albeit the mature seeds lose water. GSS also lowers both grain yield and grain quality and interferes with machine harvesting (Ciampitti 2016). Drought stress induces GSS, and an increase in the phytohormone cytokinin is observed in the source organ during seed filling (Sato et al. 2007). This syndrome induced by biotic stress such as insect damage is also caused by disturbance of the remobilization of photosynthetic products and water from leaves and stems to seeds (Hobbs et al. 2006; Hill et al. 2013; Islam et al. 2017). One of the factors for GSS caused by biotic and abiotic stresses suggests lowering sink organs. Indeed, removal of half or all pods at the R5 stage enhances GSS (Nang et al. 2011). At this stage, leaves cannot generate adequate photosynthates to keep up with the high demand from developing seeds, so the plants begin to translocate photosynthates from the lower leaves to developing seeds (Fig. 13.2a). Starting at R5, the water content of the seeds decreases in both the intact control and 50% depodded plants, while that in the stem and leaves does not decrease in the latter, indicating that GSS and a marked increase in dry weight of individual seeds occurred in depodded plants (Nang et al. 2008). During leaf senescence, cellular components such as proteins, lipids, and nucleic acids are degraded, and the released nutrients are transported to the seeds (Noodén et al. 1997; Quirino et al. 2000). The extent and rate of protein turnover are controlled by both synthesis and degradative processes such as autophagy (Kirisako et al. 1999; Nakatogawa et al. 2007). The transient upregulation of *ATGs* in seeds and the decline in leaf N content occur simultaneously (Xia et al. 2012).Total N content of leaves in the control soybean plants rapidly decreased, while leaves showing GSS gradually decreased at 4–5 weeks from R5. Expression of *GmATG8c* and *GmATG8i* in the control leaves specifically peaked at 4 weeks, while the expression of *GmATG8c* in the leaves showing GSS did not increase at the same stage (Nang et al. 2011). Therefore, autophagy is involved in GSS of soybean plants.

Furthermore, red light treatment significantly suppresses seed and pod growth in soybean, but it markedly promotes the elongation of cowpea pods (Tanaka et al. 2017). Interestingly, there are distinct differences in the pod location between soybean and cowpea: soybean pods are located at individual nodes behind the leaves (Fig. 13.2a) where they are in shade, whereas cowpea flower stalks elongate after pod setting, placing pods outside of the leaves, exposed to sunlight during seed maturation (Fig. 13.1a, b). These morpho-

logical and physiological features suggest that light quality also regulates grain yield and may be used in agronomic practices to improve increase yield of legume crops.

13.3 Remobilization of Carbohydrates and Water in Sink and Source Organs of Cereal Crops Under Environmental Stresses

13.3.1 Remobilization of Carbohydrates in Developing Seeds of Rice Exposed to Temperature Stress

World rice (*Oryza sativa*) production in 2016/2017 stands at 499.3 million tons (FAO 2017). Whole grain rice known as brown rice contains carbohydrates (74.3%), protein (6.8%), and total lipids (2.7%) with 14.9% water (MEXT 2017, Fig. 13.3). Water loss in rice grain is enhanced by high temperature during the early grain-filling stage (Funaba et al. 2006; Tanaka et al. 2009), reducing yield and grain quality (Morita et al. 2005; Tanamachi et al. 2016). High temperature causes the starch granules to be loosely packed, decreases kernel weight, and thus increases the occurrence of abnormal and chalky kernels (Resurreccion et al. 1977; Lisle et al. 2000; Tanamachi et al. 2016). Under high temperatures during grain filling, starch-hydrolyzing enzymes such as α-amylase are upregulated, while many enzymes involved in endosperm starch synthesis are downregulated (Yamakawa et al. 2007; Sreenivasulu et al. 2015, Kaneko et al. 2016). High temperatures significantly repress the expression of the sucrose transporter-coding gene *OsSUT1* in flag leaves and stems (source organs) and in seeds (sink organ) in the heat-sensitive cultivar "Hinohikari," which is widely grown in the western part of Japan (Phan et al. 2013). In addition, marked downregulation of the expression of the starch biosynthesis-related genes *OsSuSy2*, *OsAGPS2b*, *OsBEIIb*, and granule-bound starch synthase-coding genes, *OsGBSSI* in grains is induced by high temperatures at the early ripening stage,14 days after flowering (DAF) (Fig. 13.3b). By reducing the expression of *OsSUT1* and the starch synthase-related genes, high temperatures during grain filling accelerate ripening due to a hastened or premature assimilate supply to seeds accompanied by water loss and eventually reduce the dry weight and quality of harvested grains. In contrast, heat-tolerant rice cultivars have more effective sugar transport and starch accumulation, and thus they maintain high grain quality at high temperatures (Miyazaki et al. 2013). Recently, heat-tolerant cultivar "Genkitsukushi" has been bred in Fukuoka Prefecture, Japan, and the individual traits that allow them to cope with high temperature are clearly different (Miyazaki et al. 2013). The heat-tolerant cultivars markedly decrease the content of nonstructural carbohydrates (simple sugars, fructan, starch, etc.) in the stem, which functions as a source organ under high temperatures, indicating that this common trait provides heat tolerance, while there are no changes in a heat-sensitive cultivar "Hinohikari" during the early grain-filling stage. The expression of *AGPS2b*, which encodes a rate-limiting enzyme in starch synthesis, in heat-tolerant cultivars does not decrease under high temperatures (Tanamachi et al. 2016). Additionally, the expression of *Amy3E*, a starch-degradation-related gene considered to induce grain chalkiness, and in heat-tolerant cultivars such as "Genkitsukushi" is not increased by high temperatures. Notably, seeds of the several heat-tolerant cultivars maintain the nucellar epidermis, which functions in sucrose transport at the early seed development stage but which disappears in a heat-sensitive cultivar under high temperatures (Tanaka et al. 2009, Tanamachi et al. 2016).

Yield and quality of rice grains are also decreased by low temperature (Funaba et al. 2006) and low irradiance (shading stress) at the early ripening stage, owing to the suppression of *OsSUT1* expression in sink and source organs as well as a shortage of leaf-derived photoassimilates (Ishibashi et al. 2014). The above data indicate that environmental stresses such as inadequate tem-

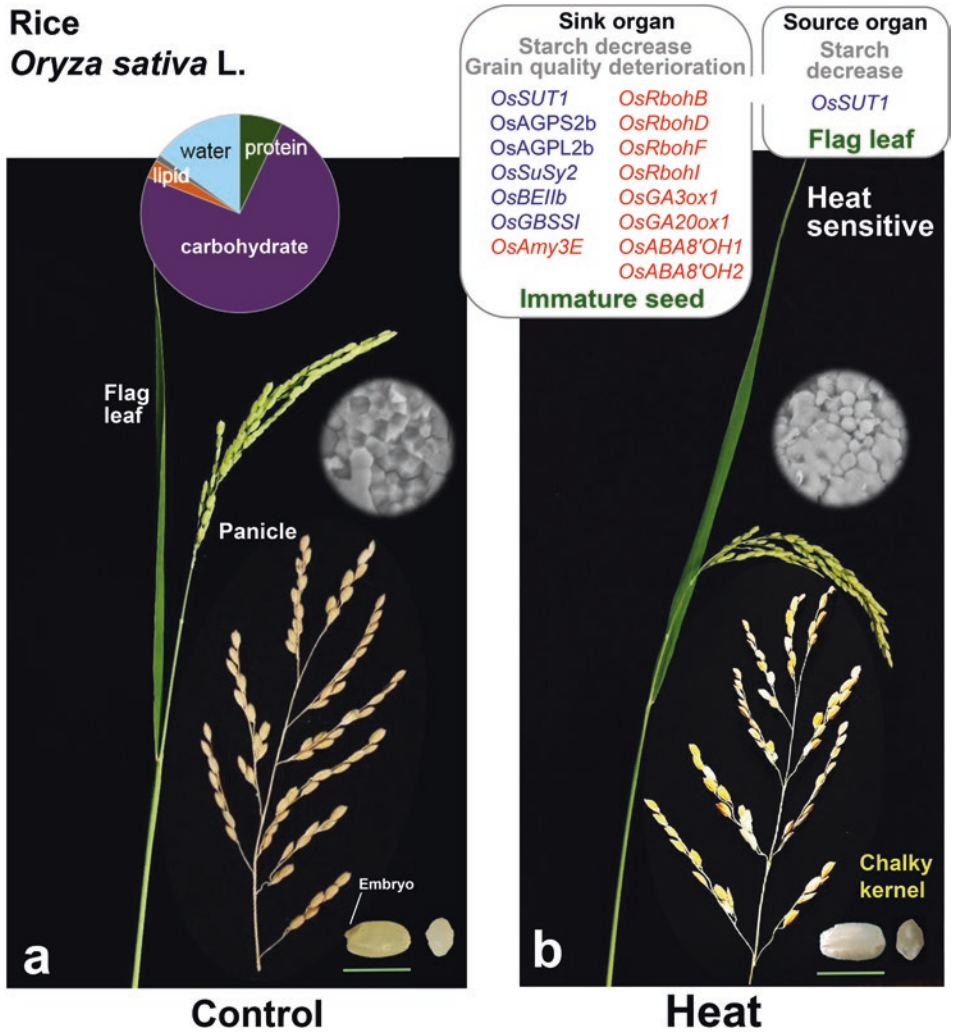

Fig. 13.3 Effect of heat stress on rice and changes in ROS-related gene expression, GA-/ABA-related gene expression, and carbohydrate-related gene expression in sink (seed) and source (flag leaf). (**a**) An immature panicle with flag leaf of rice plant under control conditions (25 °C) at 14 DAF and mature panicle (lower) at harvest stage under the control conditions. Whole grain of brown rice, cross-section of a perfect kernel, and scanning electron microscope (SEM) image of endosperm at harvest stage are also shown. (**b**) An immature panicle of rice plant with flag leaf grown at 30 °C at 14 DAF and mature panicle with sterile grains (lower) at harvest stage under the heat stress. Whole grain, cross-section of chalky kernel (back white kernel) and a SEM of endosperm in chalky grains are shown. Diameter of SEM image is 30 μm, respectively. Scale bar of seeds indicates 5 mm. Rice plants are of heat-sensitive "Hinohikari," widely cultivated in western part of Japan. Red letters indicate upregulated genes, while blue letters indicate downregulated ones induced by high temperature at 14 DAF. *Os Oryza sativa*, *SUT* sucrose transporter, *AGPS* ADP-glucose pyrophosphorylase, *BE* branching enzyme, *GBSS* granule-bound starch synthase, *Amy3E* amylase, *RbohB* respiratory burst oxidase homolog, *GA3ox1* gibberellic acid 3-*oxidase*, *ABA8′OH* abscisic acid *8′* hydroxylase. Based on information in Yamakawa et al. (2007), Phan et al. (2013), Tanamachi et al. (2016), and Suriyasak et al. (2017), mature seed content of brown rice shown in a circle graph is based on "Standard tables of food composition in Japan, #01080" (MEXT 2017). (Photograph courtesy of Chetphilin Suriyasak, Koichiro Tanamachi and Eriko Takeda, Kyushu University)

peratures and low irradiance restrict assimilate supply by suppressing the expression of *OsSUT1* and starch synthesis-related genes, resulting in deterioration of both the quality and quantity of rice grains. Furthermore, in rice grains exposed to high temperature, an increase in the expression of *NADPH oxidase* genes (such as those of the *Rboh* family) and therefore in the reactive oxygen species (ROS) content increases the expression of GA biosynthetic genes (*OsGA3ox1*, *OsGA20ox1*), ABA catabolic genes (*OsABA8'OH1*, *OsABA8'OH2*), and a starch catabolic gene, *OsAmy3E* (Fig. 13.3b) (Suriyasak et al. 2017). These data indicate that ROS generated under heat stress induce α-amylase production in maturing rice grains through the metabolism of gibberellin (GA) biosynthesis and ABA degradation and consequently cause grain chalkiness.

13.3.2 Water Status of Seeds Exposed to Environmental Stresses

As seeds develop under environmental stresses, the transport of assimilates is closely linked to water movement from source organs to seeds (Nang et al. 2008; Egashira et al. 2016). Because water is one of the factors that limit seed development, seeds must have mechanisms that allow them to withstand the loss of water with maturation (Battaglia and Covarrubias 2013; Delahaie et al. 2013). During seed maturation, cellular viscosity increases dramatically, and the cytoplasm transforms into a "glassy state" (Weng et al. 2016). According to Leprince and Walters-Vertucci (1995), a glass is defined "as an amorphous metastable state that resembles a solid, brittle material, but it retains the disorder and physical properties of the liquid state." Water proton signals in NMR spectra indicate that the number of Gaussian peaks is higher and that of Lorentzian peaks is lower in mature than in immature seeds (Iwaya-Inoue et al. 2001). The Gaussian curve is thought to correspond to loosely bound water or bound water restricted its mobility in biological systems, and the Lorentzian curve corresponds to free water in biological systems or non-biological solutions (Kockenberger et al. 1997; Pu et al. 2013). The change in water compartments and the loss of water mobility in ripening seeds are considered to reflect cellular heterogeneity such as protein, lipid, and carbohydrate accumulation at the development and ripening stages (Ishida et al. 2000; Garnczarska et al. 2007; Tanaka et al. 2009; Krishnan et al. 2014; Watanabe et al. 2015). NMR imaging (MRI) indicated by the signal of water proton density in an embryo is highly maintained compared to that of the endosperm in ripening rice seeds during 20–45 DAF (Horigane et al. 2001). A linear relationship has been established between the logarithms of rotational motion and the aging rates or longevity in dry anhydrobiotes (Buitink and Leprince 2004, 2008; Leprince et al. 2017). The NMR spin-lattice relaxation times of water protons (T_1) in rice seeds are closely related to the water quantity until the mid-maturation stage, whereas the spin-spin relaxation time (T_2) is a more sensitive indicator of the accumulation of dry matter after the mid-maturation stage (Funaba et al. 2006). Under heat stress, a lower water content around the center of endosperm was observed on MRI at an early maturation stage compared to control ones (Ishimaru et al. 2009). Hot and dry wind strongly induced osmotic stress in the endosperm of a heat-sensitive cultivar, leading to chalky ring formation in rice grains (Wada et al. 2014). In contrast, heat stress affected neither the water content and physical states of water nor starch accumulation in grains of heat-tolerant cultivars during 14 DAF, the early maturation stage (Tanaka et al. 2009). In other words, heat-tolerant cultivars have a desiccation avoidance trait and are thus capable of surviving the removal of their cellular water at the early maturation stage.

Besides, adverse environmental conditions such as long periods of rainfall or even a humid environment during grain development are generally associated with higher levels of seed germination upon grain maturation, and this predisposes plants to preharvest sprouting (PHS) (Gubler et al. 2005; Bewley et al. 2013; Fig. 13.4e). Generally, seeds do not germinate while they remain on the maternal plant; however, continual rain frequently causes vivipary,

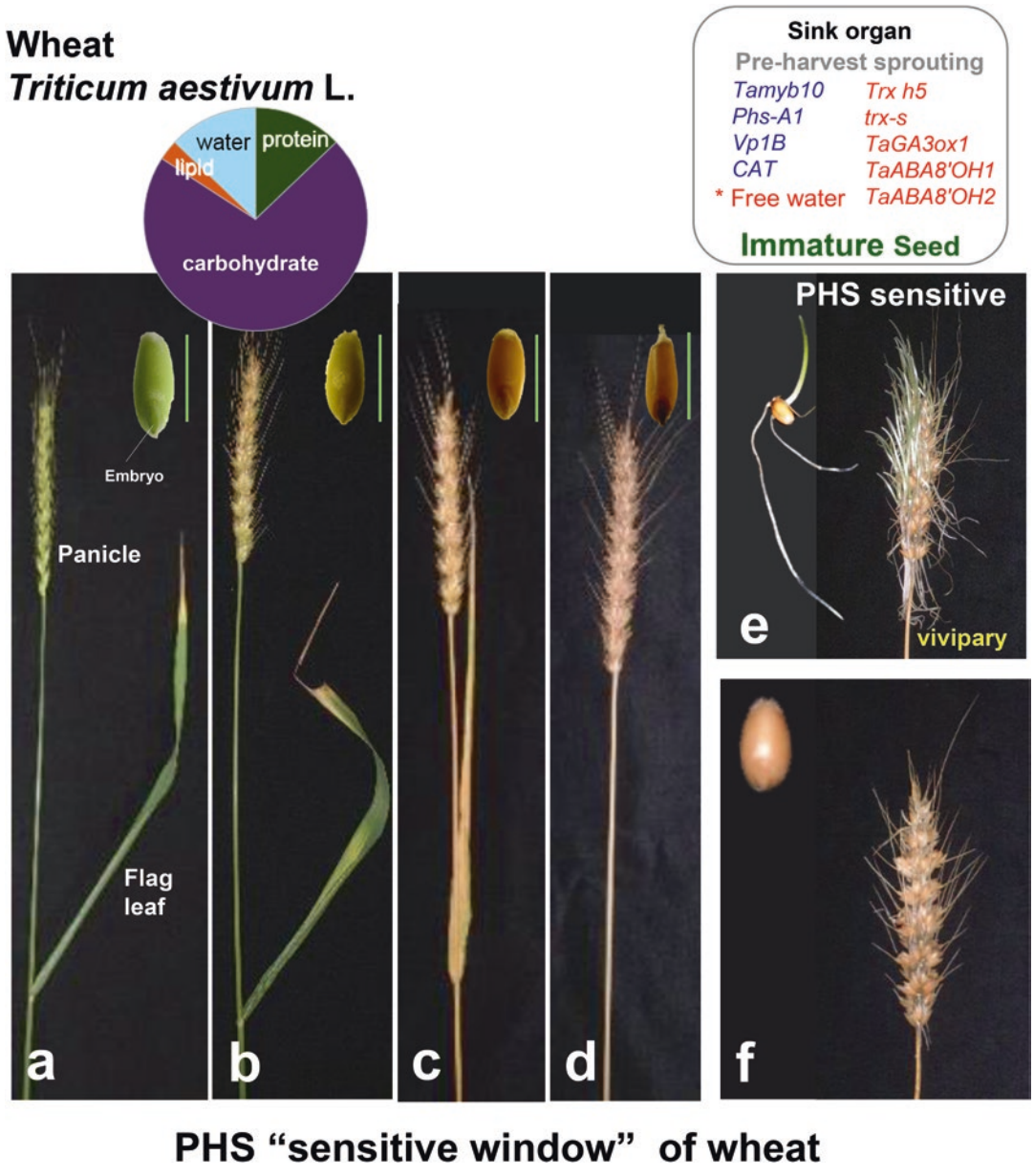

Fig. 13.4 Effect of water stress on preharvest sprouting (PHS) "sensitivity window" in wheat and changes in expression of ROS-related gene and GA-/ABA-related gene and water mobility. (**a**–**d**) A wheat panicle of PHS "sensitivity window" stage under control conditions. Panicles and developing seeds in growth stages; (**a**) 21 DAF, (**b**) 28 DAF, (**c**) 35 DAF, and (**d**) 42 DAF, respectively. (**e**) A panicle and seed showing PHS (vivipary) sprayed with distilled water for 8 days. (**f**) A panicle and seed sprayed with 50 μM ABA for 8 days. Plants are the PHS-sensitive "Shirogane-Komugi" widely cultivated in the western part of Japan. Red letters indicate upregulated genes, while blue letters indicate downregulated ones induced by distilled water treatment. *Free water written in red letter indicates that free water was kept until 28 DAF (**a**, **b**) and was 14 days longer than a PHS-resistant "Norin 61," popular cultivar in Japan. Scale bar of seed indicates 5 mm. *Ta Triticum aestivum, trx* thioredoxin, *myb10* myeloblastosis, *Phs* preharvest sprouting, *Vp* viviparous, *CAT* catalase, *GA3ox* GA3-*oxidase*, *ABA8′OH* abscisic acid *8′*hydroxylase. Based on information in Bailey et al. (1999), Ishibashi and Iwaya-Inoue (2006), Ishibashi et al. (2008), Xia et al. (2009), Himi et al. (2011), Tanaka et al. (2012), Li et al. (2009), Kashiwakura et al. (2016), Liu et al. (2016), Shorinola et al. (2016), and Zhou et al. (2017). Mature seed content of common wheat shown in a circle graph is based on "Standard tables of food composition in Japan #01012" (MEXT 2017). (Photograph courtesy of Miho Tanaka, Kyushu University)

especially to serious problem in cereals such as wheat and barley (Gubler et al. 2005; Simsek et al. 2014; Mares and Mrva 2014; Zhou et al. 2017). The world wheat (*Triticum aestivum*) production in 2016/2017 is 760.1 million tons (FAO 2017), and in exceptional years, a decrease in grain weight causes low quality and yield losses of up to 50%. A wheat seed mostly contains carbohydrates (72.2%), protein (10.6%), lipid (3.1%), and water (12.5%) (MEXT 2017; Fig. 13.4). As the seed germinates, starch and protein are degraded, severely reducing the quality of flour. Therefore, PHS resistance is one of the most important breeding objectives. Water content in developing grain is used as a conventional index of PHS. By contrast, there were no differences in the water contents in each stage among wheat cultivars with different PHS resistances, while seeds of the PHS-sensitive cultivar "Shirogane-Komugi" kept free water until 28 DAF indicating PHS-"sensitive window" (Fig. 13.4a, b), 14 days longer than in the resistant cultivar "Norin 61"; there were no differences in T_1 between the two cultivars (Tanaka et al. 2012). In other words, seeds of "Norin 61" mainly consist of loosely bound water or bound water (as indicated by T_2) on and after 14 DAF. The term "bound water" has been used to describe the restricted mobility of water molecules remaining after a material has been partially dried, and the properties of bound water depend on liquid water molecules because of short-range interactions in the vicinity of molecular surfaces (Murase and Watanabe 1989; Wolfe et al. 2002). The formation of intracellular glasses is indispensable for seeds to survive a dry state. Indeed, the storage stability of seeds is related to the packing density and molecular mobility of the intracellular glass, suggesting that the physicochemical properties of intracellular glasses provide stability for long-term survival (Buitink and Leprince 2008). During seed maturation, viscosity dramatically increases, and thus the cytoplasm transforms into a "glassy state" in a cell. As a result, it suggests that the seeds of PHS-resistant wheat cultivar also have a glassy trait at early maturing stage.

In wheat, *Vp1B* and *Phs-A1* confer resistance to PHS by affecting the rate of dormancy loss during dry seed after-ripening (Xia et al. 2009; Shorinola et al. 2016). PHS damage is due mainly to α-amylase activity, which can be characterized by a low falling number (Imabayashi and Ogata 1998). The PHS trait is affected by ABA biosynthesis and the expression of catabolism-related genes in wheat and barley seeds (Gubler et al. 2005; Chono et al. 2006; Bewley et al. 2013; Shorinola et al. 2016). Effect of ABA treatment on suppressing wheat PHS is shown in Fig. 13.4f, while marked PHS is observed in panicle sprayed with distilled water (Fig. 13.4e). Regulatory mechanism influencing the expression of ABA catabolism-related genes such as *TaABA8'OH1*, *TaABA8'OH2*, and GA biosynthesis gene, *TaGA3ox1* was critical for dormancy maintenance and breakage at low temperatures (Kashiwakura et al. 2016). Studies combining a noninvasive analysis and a molecular approach will help solve this PHS problem for Poaceae (Rolletschek et al. 2011, 2015; Melkus et al. 2011; Pielot et al. 2015).

Moreover, white wheats kernel such as "Shirogane-Komugi" are usually more susceptible to PHS than red grain wheats such as "Norin 61" (Tanaka et al. 2012). This association between PHS resistance and red pigmentation is likely due to a pleiotropic effect of the genes controlling grain color. Wheat color genes of red grain (*R-1*) encode a Myb transcription factor (*TaMyb10*) regulates flavonoid biosynthesis (Himi et al. 2011; Zhou et al. 2017). Himi et al. (2011) indicated that product of *Tamyb10-D1* of red grain cultivar can induce the expression of flavonoid biosynthesis-related genes which regulate anthocyanin synthesis. Anthocyanins scavenge the free radicals and ROS. Indeed, expression of the catalase (*CAT*) gene and CAT activity in seeds are higher in PHS-resistant, red grain cultivar than in the white grain cultivar at 28–42 DAF; thus, the ability to scavenge H_2O_2, one of ROS, is high in PHS-resistant cultivars (Ishibashi and Iwaya-Inoue 2006; Ishibashi et al. 2008). Thioredoxin (Trx) is also a key antioxidant system in defense against oxidative stress through its disulfide reductase activity. In contrast, it is particularly worth-noting that overexpression of *trx-h* gene in the endosperm of barley (*Hordeum vulgare*) seeds led to an increase in activities of α-amylase and starch debranching enzyme, as a result accelerated

germination (Cho et al. 1999; Wong et al. 2002). Moreover, their group indicated that overexpression of *Trx h5* in the starchy endosperm also showed accelerated wheat seed germination, while a transgenic wheat underexpressing *trx h9* gene has shown outstanding PHS resistance (Li et al. 2009). In addition, wheat seeds with antisense thioredoxin-s (*anti-trx-s*) gene expression indicated highly PHS resistance (Guo et al. 2011). Anti-*trx-s* inhibited the endogenous *trx-h* expression and lowered α-amylase activity resulting in high PHS resistance in the transgenic wheat. Interestingly, anti-*trx-s* lowered the overall metabolic activities of mature seeds removing preharvest sprouting potential, while postharvest ripening reactivated the metabolic activities of the transgenic seeds to restore the germination ability in wheat (Liu et al. 2016). They indicated that the readjustment of metabolic activities in wheat seeds is also critically important to break seed dormancy and germination.

13.4 Do ROS Produced in Seeds After Imbibition Cause Oxidative Stress or Act as Signaling Molecules During Seed Germination?

13.4.1 Induction of ROS in Seeds After Imbibition

Moisture content in most mature seeds is much lower than in other plant organs; this low content ensures seed viability during long-term storage. However, seeds need to absorb a considerable amount of water in order to germinate (Pietrzak et al. 2002). Phase I of the germination process is initiated by imbibition, which is required to activate the respiratory metabolism and transcriptional and translational activities. In phase II, known as germination sensu stricto, water uptake ceases and reserve mobilization starts. In phase II, plant hormones such as GAs, ABA, and ethylene, which play key roles in seed dormancy and germination, are regulated. ABA is the key molecule in the induction and maintenance of dormancy (Finkelstein et al. 2008; Rodríguez et al. 2015; Shu et al. 2016). In contrast, GA and ethylene promote seed germination via a complex signaling network. Phase III is characterized by radicle protrusion (Bewley and Black 1994; Bewley 1997; Nonogaki et al. 2010; Weitbrecht et al. 2011). Germination requires specific temperatures, oxygen levels, and light, with the exact proportions being species specific (Corbineau et al. 2014).

In general, ROS such as O_2^-, hydrogen peroxide (H_2O_2), and •OH cause oxidative damage to lipids, proteins, and nucleic acids. Indeed, seed deterioration during storage is due in part to the peroxidation of membrane lipids by ROS and the resulting leakiness of the membranes (Sung and Jeng 1994; Bailly et al. 1998). Seed longevity is enhanced through the elimination of ROS by overaccumulated ROS scavengers in transgenic seeds (Lee et al. 2010; Zhou et al. 2012). However, they also play various important roles in cellular signaling in plants, notably acting as regulators of growth and development, programmed cell death, hormone signaling, and responses to biotic and abiotic stresses (Mittler et al. 2004). In seed physiology, several studies have reported that exogenous H_2O_2 promotes seed germination in many plants (Chien and Lin 1994; Fontaine et al. 1994). Furthermore, the production of H_2O_2 during the early imbibition period has been demonstrated in seeds of soybeans (Puntarulo et al. 1991), maize (Hite et al. 1999), wheat (Caliskan and Cuming 1998), barley (Ishibashi et al. 2010), and *Zinnia elegans* (Ogawa and Iwabuchi 2001). The localization of mRNA for NADPH oxidase, which is one of the major sources of ROS, in scutellar epithelial and aleurone cells in imbibed barley seeds is consistent with the production of O_2^- and H_2O_2 (Fig. 13.5a–c) (Ishibashi et al. 2015). In sunflower and soybean, H_2O_2 production is accelerated in the embryonic axis of seeds after imbibition (Fig. 13.5e, f) (Oracz et al. 2007; Ishibashi et al. 2013). Therefore, ROS produced after imbibition appear to regulate seed germination. Indeed, in barley seeds, NADPH oxidase acts as a key enzyme in germination and subsequent seedling growth (Ishibashi et al. 2010). In pea seeds, H_2O_2 accelerates germination and stimulates the early growth of seedlings (Barba-Espin et al. 2010). In contrast, exogenous antioxidants, which act as ROS scavengers, significantly suppressed seed germination in

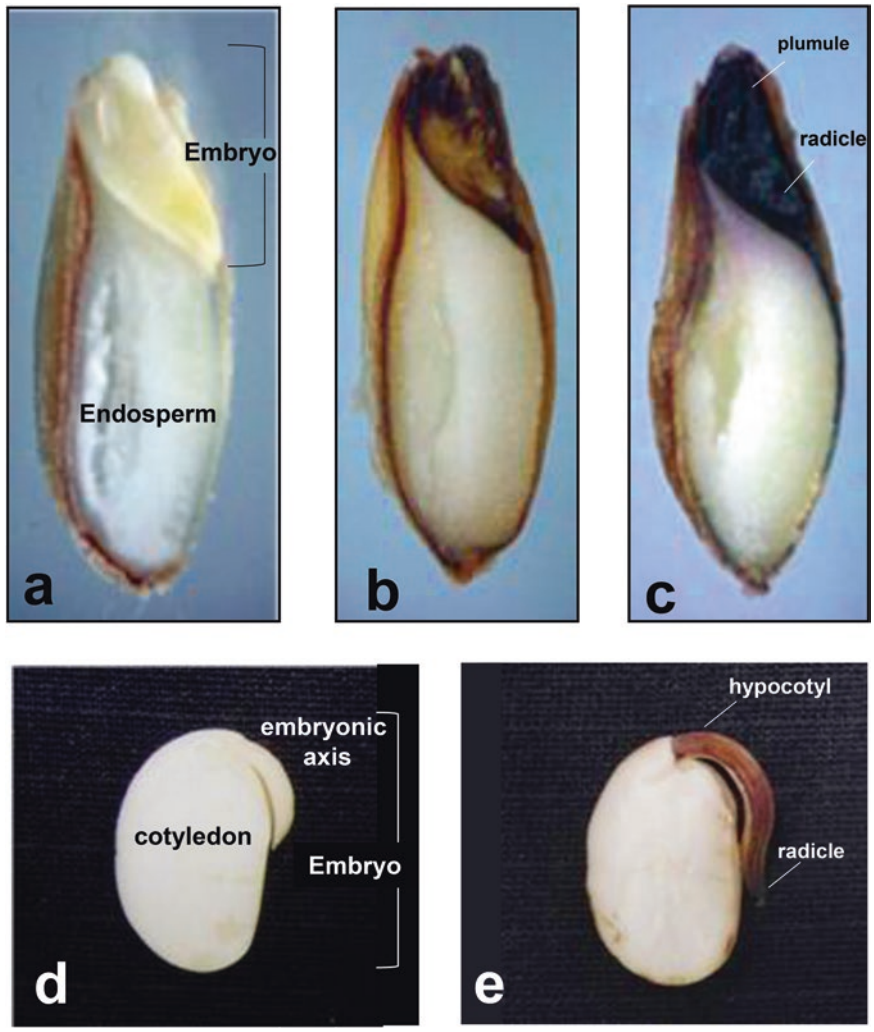

Fig. 13.5 The production of ROS in imbibed barley and soybean seeds. (**a**) No staining in a barley seed. (**b**) Staining with nitro blue tetrazolium in a barley seed. (**c**) Staining with 3,3-diaminobenzidine in a barley seed and (**d**) that of a soybean seed. Barley seeds (**a**–**c**) were treated with distilled water for 24 h. Soybean seeds were treated with (**d**) distilled water or (**e**) 25 mM *N*-acetylcysteine for 24 h. Based on information in Ishibashi et al. (2013, 2015) (Reproduced with permission from Ishibashi et al. 2013, 2015, respectively)

several species (Ogawa and Iwabuchi 2001; Ishibashi and Iwaya-Inoue 2006).

13.4.2 Oxidative Window for Seed Germination

Recently, it was reported that several signaling molecules such as nitric oxide and ROS also regulate seed dormancy and germination (Ma et al. 2016). In barley, ROS break seed dormancy and promote germination (Fontaine et al. 1994; Bahin et al. 2011; Ishibashi et al. 2017). The relationships among ROS, seed dormancy, and germination have been described for many plant species, including *Z. elegans* and sunflower (Ogawa and Iwabuchi 2001; Oracz et al. 2007). H_2O_2 is regarded as a signaling hub for the regulation of seed dormancy and germination; the precise regulation of H_2O_2 accumulation by the cell antioxidant machinery is essential to achieve a balance between oxidative signaling that promotes

germination and oxidative damage that prevents or delays germination (Wojtyla et al. 2016). These findings were clearly summarized and presented by Bailly et al. (2008) as the principle of the "oxidative window" for germination. According to this hypothesis, both lower and higher levels of ROS impair seed germination, which is only possible within a defined range of concentrations.

13.4.3 Regulation of ROS Metabolism in Seeds by Plant Hormones

The induction of ROS in seeds after imbibition is regulated by plant hormones. ABA treatment reduces the O_2^- and H_2O_2 content in embryos of barley seeds as well as the expression of most *NADPH oxidases* (*HvRbohB1*, *HvRbohE*, *HvRbohF1*, and *HvRbohF2*, but not *HvRbohB2*) and NADPH oxidase activity. The relationship between ABA and ROS can be either antagonistic or synergistic, depending on the organ and the biological phenomenon. For example, ABA-induced stomatal closure is accompanied by ROS accumulation in guard cells owing to the activation of NADPH oxidases rbohD and rbohF (Zhang et al. 2008), and the ABA-mediated response to salt stress or drought is related to ROS production (Cruz de Carvalho 2008; Ren et al. 2012). In contrast, exogenous ABA reduces ROS accumulation in wheat leaves by stimulating antioxidant enzyme activities (Du et al. 2013). As mentioned above, most studies have shown that ROS production is suppressed by ABA in seeds (El-Maarouf-Bouteau and Bailly 2008; Ishibashi et al. 2012; Ye et al. 2012).

There have been numerous reports on the relationship between ROS and ethylene. In legumes, ROS and ethylene are part of a Nod-factor-induced signaling cascade that is important for the initiation of nodule primordia (D'Haeze et al. 2003). In winter squash (*Cucurbita maxima*), 1-aminocyclopropane-1-carboxylate synthase (*CmACS1*), a key enzyme in the regulation of ethylene production, is inhibited by diphenyleneiodonium, which blocks the superoxide-generating enzyme NADPH oxidase (Watanabe et al. 2001). In mung bean hypocotyls, exogenous 1-aminocyclopropane-1-carboxylic acid did not affect ROS production, but hypocotyls exposed to H_2O_2 showed high ethylene accumulation as a result of the activation of ethylene biosynthesis enzymes (Song et al. 2007). In sunflower seeds, ethylene induces O_2^- and H_2O_2 in the embryonic axis after imbibition (El-Maarouf-Bouteau et al. 2015). ROS produced during soybean seed imbibition promote ethylene production through the expression of *GmACS2e* and *GmACS6a* (Ishibashi et al. 2013).

In barley aleurone cells, GA increases the activity of NADPH oxidases, whereas ABA suppresses this activity and induces the production of O_2^- and H_2O_2 (Ishibashi et al. 2015). Gilroy (1996) reported that GA increases Ca^{2+} and calmodulin levels in barley aleurone cells, that ABA antagonizes this effect, and that the regulation of cytoplasmic Ca^{2+}, and calmodulin is important in the secretion of enzymes such as α-amylase by barley aleurone cells. In addition, the activation of NADPH oxidases depends on the influx of Ca^{2+} into the cytoplasm and on the phosphorylation of the N-terminal regions of these enzymes by a Ca^{2+}-dependent protein kinase, because the N-terminal region contains regulatory elements such as calcium-binding EF-hands and phosphorylation domains (Suzuki et al. 2011). In barley aleurone cells, $LaCl_3$ and calmidazolium markedly suppressed the GA-induced increase in NADPH oxidase activity. An increase in Ca^{2+} was suggested to be an immediate response of the barley aleurone layer to GA (Bethke et al. 1997). These data indicate that the increase in Ca^{2+} induced by GA in aleurone cells activates NADPH oxidases (Ishibashi et al. 2015).

13.4.4 ROS Signaling During Seed Germination

The breaking of dormancy by ROS has been reported in relation to plant hormone signaling in seeds of several species (El-Maarouf-Bouteau et al. 2013). In germinated seeds, exogenously applied ABA inhibits ROS accumulation in barley

(Ishibashi et al. 2012), rice (Ye et al. 2012), lettuce (Zhang et al. 2014), and sunflowers (El-Maarouf-Bouteau et al. 2015). By contrast, the addition of GA enhances the production of ROS, mainly superoxide and H_2O_2, in radish (Schopfer et al. 2001), and *Arabidopsis* (Liu et al. 2010; Lariguet et al. 2013). Bahin et al. (2011) suggested that exogenously applied H_2O_2 does not influence ABA biosynthesis or signaling but has a pronounced effect on GA signaling, resulting in a shift in hormonal balance and in the initiation of germination. The modulation of the phytohormone balance during germination by exogenously applied H_2O_2 is also a product of changes in H_2O_2 levels in seeds treated with GA and ABA. Exogenous H_2O_2 and a NADPH oxidase inhibitor increase ABA catabolism by enhancing the expression of *CYP707A* genes, which encode ABA 8′-hydroxylases and enhance the expression of genes for GA synthesis in dormant *Arabidopsis* seeds (Liu et al. 2010) and nondormant barley seeds (Ishibashi et al. 2015). In nondormant barley seeds, H_2O_2 accumulation via superoxide production by NADPH oxidases promotes GA biosynthesis in embryos; the resulting GA induces and activates NADPH oxidases in aleurone cells, and accumulated H_2O_2 induces α-amylase in these cells (Ishibashi et al. 2015). In barley aleurone cells, H_2O_2 release the repression of GA-regulated Myb transcription factor (GAMyb) mRNA by ABA-responsive protein kinase (PKABA) and consequently promote the production of α-amylase mRNA, thus suggesting that the H_2O_2 generated in aleurone cells in response to GA antagonizes ABA signaling (Fig. 13.6) (Ishibashi et al. 2012). In barley, however, the embryos of

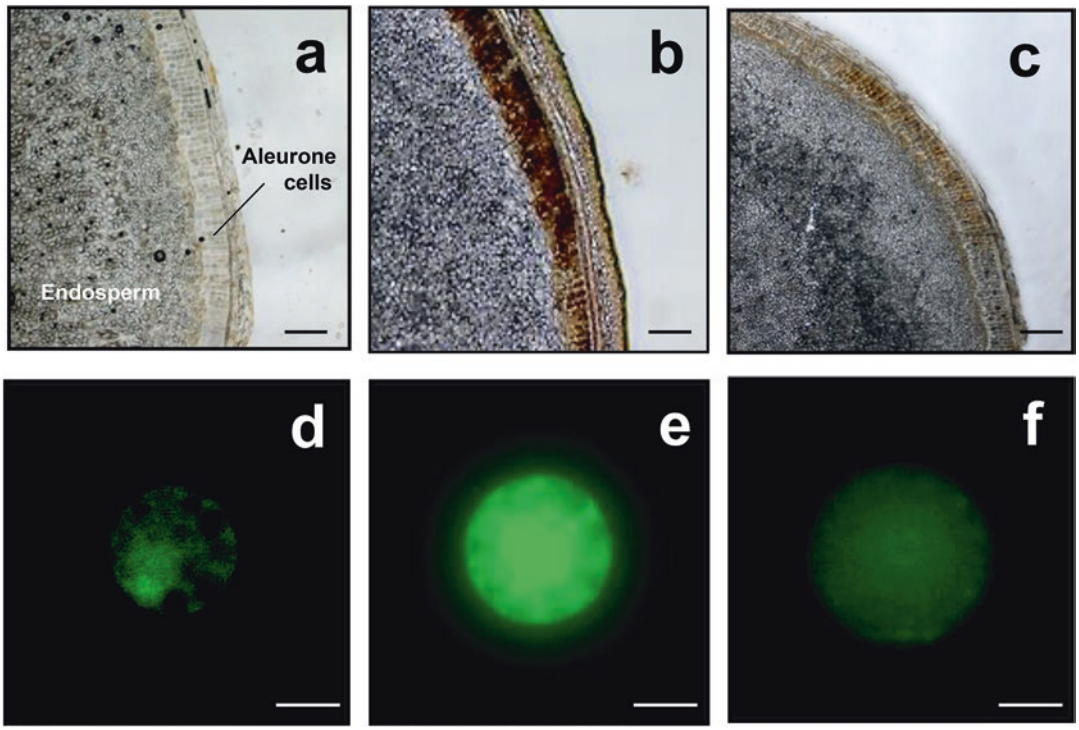

Fig. 13.6 Production of H_2O_2 in barley aleurone cells treated with phytohormones. An embryoless half-seed was treated with (**a**, **b**) 1 μM GA or (**c**) 1 μM GA + 5 μM ABA for 24 h, then incubated (**a**) without or (**b**, **c**) with 3,3-diaminobenzidine for 1 h. (**d**–**g**) Visualization of H_2O_2 in aleurone protoplasts loaded with dihydrofluorescein diacetate. Protoplasts were incubated with (**d**) no hormone as a control, (**e**) 1 μM GA, or (**f**) 1 μM GA + 5 μM ABA for 24 h at 22 °C. Scale bars, (**a**–**c**) 250 μm or (**d**–**f**) 25 μm. Based on information in Ishibashi et al. (2012). (Reproduced with permission from in Ishibashi et al. 2012)

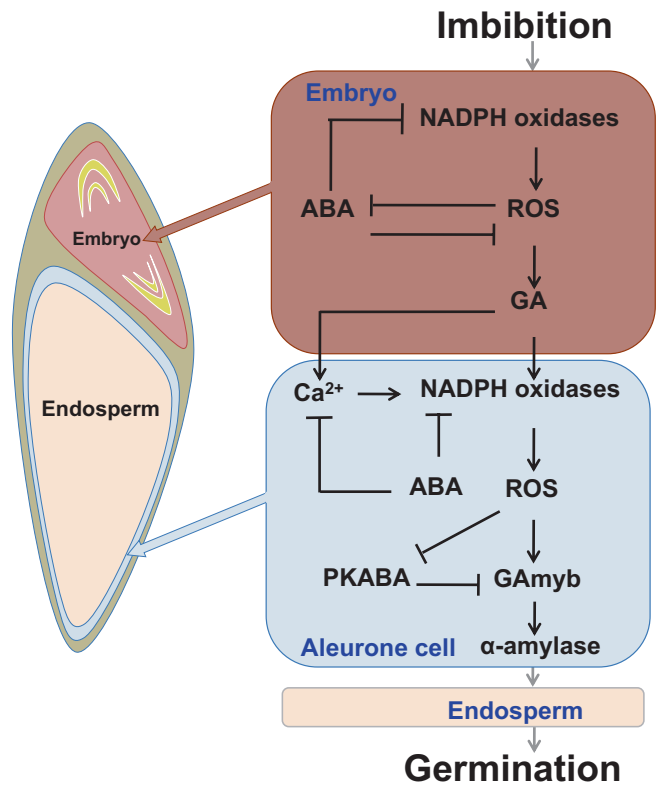

Fig. 13.7 Role of ROS in barley seed germination and dormancy. After imbibition, ROS produced by NADPH oxidases promote GA biosynthesis and ABA catabolism in germinating embryos. In aleurone cells, ROS generated in response to GA induce *GAMyb* mRNA by suppressing the gene expression and activity of PKABA; in this way, ROS promote the expression of α-amylase mRNA. (Based on information in Ishibashi et al. 2012, 2015, 2017)

dormant seeds maintain high ABA contents, promoting *HvCAT2* expression through HvABI5 for H_2O_2 catabolism. Because ABA catabolism through HvABA8′OH1 is promoted by H_2O_2, dormant barley seeds have low ROS contents and low *HvABA8′OH1* expression, which results in high ABA content. These data suggest that the balance between ABA and ROS changes in barley embryos after imbibition and regulates seed dormancy and germination (Fig. 13.7) (Ishibashi et al. 2017). Ethylene alleviates sunflower seed dormancy, whereas ABA represses germination. Ethylene treatment induces ROS generation in the embryonic axis, whereas ABA has no effect on ROS production. The beneficial effect of ethylene on germination is lowered in the presence of antioxidant compounds, and methyl viologen, a ROS-generating compound, suppresses the inhibitory effect of ABA. Methyl viologen treatment did not alter significantly ethylene or ABA production during seed imbibition (El-Maarouf-Bouteau et al. 2015). Altogether, these data shed new light on the crosstalk between ROS and plant hormones in seed germination.

Other evidence shows that the selective oxidation of proteins and mRNAs can promote seed germination (Job et al. 2005; Oracz et al. 2007; Barba-Espín et al. 2010; Bazin et al. 2011). Protein oxidation can alter protein functions as a result of modifications made to their enzymatic and binding properties (Davies 2005). Indeed, H_2O_2 accumulation and the associated oxidative damage together with a decline in antioxidant mechanisms can be regarded as a source of stress that may affect the successful completion of germination. However, H_2O_2 is also regarded as a signaling hub for the regulation of seed dormancy and germination, and the precise regulation of H_2O_2 accumulation by the cell antioxidant machinery is crucial to achieve a balance between oxidative signaling that promotes germination and oxidative damage that prevents or delays germination. Bazin et al. (2011) showed that approximately 24 stored

mRNAs undergo oxidation during sunflower (*Helianthus annuus*) after-ripening. ROS production during germination contributes to reserve mobilization through oxidative modifications of stored proteins; storage organs may then recognize these modifications as signals for reserve mobilization to the rapidly growing axis. Due to the abundance of available seed storage proteins, the oxidized forms of these proteins, such as heat shock proteins and elongation factors, may also act in ROS signaling involved in seed germination (Job et al. 2005; Barba-Espín et al. 2010). Oracz et al. (2007) proposed a mechanism of seed dormancy release that involves a change in proteome oxidation resulting from the accumulation of ROS during the after-ripening phase.

13.5 Concluding Remarks

This review summarizes a strategy used by crop plants to control seed maturation and germination under environmental stresses, involving nutrient remobilization, water mobility, plant hormones, and ROS in sink/source organs. Cowpea is one of the most drought-tolerant crops. Despite a dramatic decrease in photosynthesis caused by rapid stomatal closure, it maintains grain yield by the translocation of photoassimilates from leaves via temporary sink/source organ, flower stalk, to seeds. In contrast, the productivity of drought-stressed soybean is limited; the suppression of biosynthesis and the promotion of degradation reduce the accumulation of lipids and proteins in maturing seeds, which are forced to accumulate sugar instead to maintain vigor. Generally, heat stress also causes grains to lose water during the early grain-filling stage. In heat-sensitive rice, grain quality and quantity are reduced by the downregulation of genes related to sucrose transporters and starch synthase with the upregulation of genes related to starch degradation. ROS produced under heat stress become involved in α-amylase induction and in the maturation of rice grains through GA/ABA metabolism, causing grain chalkiness. Although developing seeds generally do not germinate while they remain on the mother plant, a humid environment can cause PHS damage. Seeds of wheat cultivars susceptible to PHS cannot maintain a glassy state for as long as, and activities of ROS scavengers are lower than, those of resistant cultivars during the seed-maturing stage.

Seed germination is regulated by a complex signaling network. As mentioned above, ROS interact with GA, ABA, and ethylene, hormones that are essential regulators of seed dormancy and germination and that act as a central hub in the regulation of germination. These interactions are involved in the regulation of the balance of ethylene and ABA and in the homeostasis of GA. Analysis of the effects of ROS on specific cellular processes highlighted by studies of dormancy and germination, such as transcription regulation, should help to elucidate the network that regulates germination in seeds.

In conclusion, a systematic focus on nutrient remobilization in relation to water status and plant hormones in maturing and germinating seeds exposed to environmental stresses can facilitate increases in crop yields and quality and developments in plant biotechnology.

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14

The Antioxidant System in the Anhydrobiotic Midge as an Essential, Adaptive Mechanism for Desiccation Survival

Alexander Nesmelov, Richard Cornette, Oleg Gusev, and Takahiro Kikawada

Abstract

One of the major damaging factors for living organisms experiencing water insufficiency is oxidative stress. Loss of water causes a dramatic increase in the production of reactive oxygen species (ROS). Thus, the ability for some organisms to survive almost complete desiccation (called anhydrobiosis) is tightly related to the ability to overcome extraordinary oxidative stress. The most complex anhydrobiotic organism known is the larva of the chironomid *Polypedilum vanderplanki*. Its antioxidant system shows remarkable features, such as an expansion of antioxidant genes, their overexpression, as well as the absence or low expression of enzymes required for the synthesis of ascorbate and glutathione and their antioxidant function. In this chapter, we summarize existing data about the antioxidant system of this insect, which is able to cope with substantial oxidative damage, even in an intracellular environment that is severely disturbed due to water loss.

Keywords

Anhydrobiosis · *P. vanderplanki* · Antioxidant · Thioredoxin · Glutathione peroxidase · Superoxide dismutase

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Abbreviations

ARIds	Anhydrobiosis-related gene islands
CCS	Copper chaperone protein
GPx	Glutathione peroxidase
Grx-like	Glutaredoxin-like
MPEC	2-Methyl-6-p-methoxyphenylethynylimidazopyrazinone
ROS	Reactive oxygen species

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SOD	Superoxide dismutase
TRX	Thioredoxin
TrxR	Thioredoxin reductase

14.1 Introduction

Once acquired, the ability to use free oxygen opened a multitude of possibilities for increasing the size, complexity, and diversity of living organisms throughout the course of evolution. However, oxygen can have a severe negative impact on aerobic organisms. Reactive oxygen species (ROS) are continuously generated inside aerobic cells and are tightly related to chronic inflammation, cancer, neurodegenerative diseases, and aging (Finkel and Holbrook 2000; Uttara et al. 2009; Reuter et al. 2010). Thus, adaptation to aerobic metabolism required the development of effective antioxidant systems.

An example of the most extreme adaptation to ROS accumulation is found in organisms that are able to survive complete desiccation, so-called anhydrobiosis. Dehydration causes a dramatic increase in ROS production and related damage due to the disruption of the mitochondrial electron transport chain, reduction of the hydrating shell of macromolecules, an increase of ionic strength, and pH changes (Leprince et al. 1994; França et al. 2007). Thus, adaptation to anhydrobiosis requires the development of an unusually effective system of antioxidant defense. Indeed, evidence of enhanced antioxidant systems has been observed for several anhydrobiotic organisms, but many details of their molecular background are yet to be uncovered.

The most complex and largest anhydrobiotic animal is the larva of the African chironomid *Polypedilum vanderplanki*. The larvae inhabit rock pools in semiarid regions of Africa, such as Nigeria, that temporarily fill with rain during the rainy season but then dry completely (Hinton 1951). The larvae successfully survive desiccation and completely recover upon rehydration to continue their development. More than 90% of *P. vanderplanki* larvae revive after being dehydrated to only 3% of residual water content (Sakurai et al. 2008; Cornette and Kikawada 2011). It has been the only known insect that can withstand such desiccation, although an African species, *Polypedilum ovahimba*, from Namibia was also described as putatively anhydrobiotic (Cranston 2014). More recently, a new anhydrobiotic midge, *Polypedilum pembai*, was found in Malawi (Cornette et al. 2017). In the dry state, the larvae also can tolerate different abiotic stresses, including extreme temperature fluctuation and high doses of ionizing radiation (Hinton 1960; Watanabe et al. 2007). A slow process of desiccation, mandatory for successful induction of anhydrobiosis, is beneficial for identifying the components essential for adaptation to water loss. To date, the best characterized biomolecular contributors to anhydrobiosis include the nonreducing sugar trehalose and a set of protective proteins (Cornette et al. 2010; Gusev et al. 2014). Here, we focus on a comparative analysis of genome and transcriptome data for the *P. vanderplanki* antioxidant defense system, which contains an expanded set of related genes and regulatory elements performing desiccation-dependent gene activation. We also describe some parts of the *P. vanderplanki* antioxidant system that are composed of different proteins and discuss a role for trehalose as an antioxidant. Sharp contrasts between *P. vanderplanki* and the congeneric midge *Polypedilum nubifer* or other insects unable to survive complete desiccation shed light on the molecular basis of the antioxidant defense system, which is essential for both natural and artificial anhydrobiosis.

14.2 Gene Diversity and Genomic Organization of the *P. vanderplanki* Antioxidant System

From the *P. vanderplanki* genome, we identified 107 genes encoding proteins related to antioxidant defense (Cornette and Kikawada 2011; Gusev et al. 2014). This set is considerably expanded compared to the published genome data of insects that are sensitive to desiccation, including *P. nubifer* (70 genes), the honey bee

Table 14.1 Genes of the antioxidant system in *P. vanderplanki*. Genes lacking orthologs in a set of other insects (*A. mellifera, A. gambiae, D. melanogaster*, and *P. nubifer*) are given on the right side of the table. *P. vanderplanki* gene abbreviations are given in accordance with our database MidgeBase http://bertone.nises-f.affrc.go.jp/midgebase/

Common insect genes		*P. vanderplanki*-specific genes	
Gene name	*P. vanderplanki* gene	Gene name	*P. vanderplanki* gene
Cat	Pv.08902	*Gtpx1*	Pv.10826
CCS	Pv.08727	*Sod4, Zn/Cu*	Pv.12949
Grx1	Pv.05207	*Sod5, Zn/Cu*	Pv.11745
Grx1	Pv.16689	*Trx genes*	AB842161 (PvTrx4)
Grx2	Pv.09247		AB842159 (PvTrx5)
Grx-like1	Pv.13721		AB842160 (PvTrx6)
MsrA	Pv.10630		AB842155 (PvTrx7)
MsrB	Pv.01571		AB842153 (PvTrx8)
Rsod	Pv.05672		AB842154 (PvTrx9)
Sod1, Zn/Cu	Pv.06735		AB842152 (PvTrx10)
Sod2, Mn	Pv.16049		AB842163 (PvTrx11)
Sod3, Zn/Cu	Pv.11918		AB842148 (PvTrx12)
Sodq	Pv.04855		AB842164 (PvTrx13)
Tpx1	Pv.03504		AB842165 (PvTrx14)
Tpx2	Pv.08988		AB842166 (PvTrx15)
Tpx3	Pv.04918		AB842149 (PvTrx16)
Tpx4	Pv.08740		AB842167 (PvTrx17)
Tpx5	Pv.08109		AB842146 (PvTrx18)
Trx/Gtx	Pv.00627		AB842147 (PvTrx19)
Trx-1	AB842156 (PvTrx1-1)		AB842151 (PvTrx20)
Trx-1	AB842157 (PvTrx1-2)		AB842150 (PvTrx21)
Trx1-like	Pv.11240		AB842162 (PvTrx22)
Trx1-like	Pv.09890		AB842158 (PvTrx23)
Trx1-like	Pv.09861		AB842144 (Pvtrx24)
Trx-2	AB842145 (PvTrx2)		
Trx-3	AB842168 (PvTrx3)		
TrxR-1	Pv.12829		

(*Apis mellifera*, 35 genes), *Anopheles gambiae* (45 genes), and *Drosophila melanogaster* (58 genes) (Corona and Robinson 2006) (Table 14.1). In comparison to the *P. nubifer* genome, the *P. vanderplanki* genome contains additional genes encoding 22 thioredoxins (TRX), 15 glutathione S-transferases, 2 superoxide dismutases (SOD), and 1 gene for glutaredoxin-like (Grx-like) protein (Table 14.1).

Such an expansion of the antioxidant gene set was achieved by extensive gene duplication and divergence, which we conclude based on colocalization of most *P. vanderplanki*-specific antioxidant genes. Thus, antioxidant genes share a remarkable feature of most *P. vanderplanki*-specific genes, which is localization to compact clusters, referred to as "anhydrobiosis-related gene islands" (ARIds) (Gusev et al. 2014). Different ARIds contain sets of paralogous genes, which are mainly upregulated in response to desiccation. Antioxidant genes encoding thioredoxins provide an excellent example of such localization, as 19 out of 21 *P. vanderplanki*-specific TRX genes are located within one of the largest ARIds, ARId5 (Fig. 14.1).

Thus, the present structure of TRX-containing ARId5 illustrates extensive gene duplication and divergence as a remarkable feature of the *P. vanderplanki* genome. One putative role for such an increase in gene diversity may be related to the variability of potential targets in the cells. Moreover, the divergence of some *P. vanderplanki* gene reaches is so substantial that new

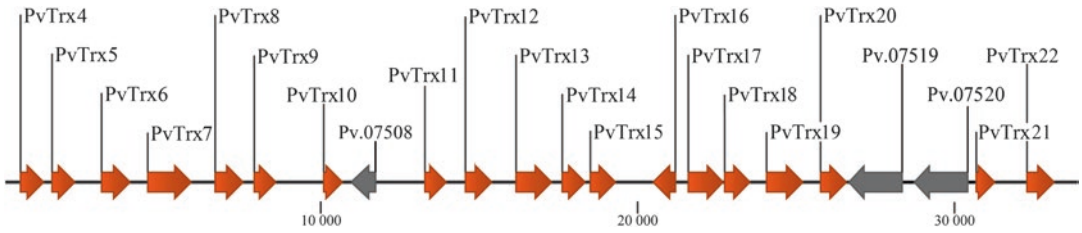

Fig. 14.1 Schematic of a part of the ARId5 region incorporating 19 TRX genes in the *P. vanderplanki* genome. TRX genes are depicted by red arrows and abbreviated in accordance with our published database MidgeBase (http://bertone.nises-f.affrc.go.jp/midgebase)

specificity or even new function can be suggested for the corresponding proteins. For example, we identified TRX genes in *P. vanderplanki* that encode proteins with previously unknown variations of typical CxxC motif (Nesmelov et al. 2016). Similarly, glutathione peroxidase (GPx) is highly expressed during desiccation in *P. vanderplanki*, with a prevalence of splice form encoding proteins that lack the catalytic glutamine residue conserved in virtually all related GPx members (Gusev et al. 2014).

14.3 Regulation of Antioxidant Genes in *P. vanderplanki*

Besides the expansion and divergence of genes, evolution in *P. vanderplanki* resulted in the acquisition of a regulatory system controlling their expression patterns. Most antioxidant system genes are upregulated in response to desiccation in *P. vanderplanki* larvae (Table 14.2). Interestingly, these upregulated genes are mostly specific to *P. vanderplanki*, with only a small number of common orthologous insect genes (Table 14.2). In a close relative of *P. vanderplanki*, the desiccation-sensitive insect *P. nubifer*, none of the antioxidant genes are upregulated in response to desiccation, including even 1:1 orthologs of some *P. vanderplanki* genes. Upregulation of antioxidant genes in *P. vanderplanki* is associated with an experimentally detectable elevation of antioxidant activity (Gusev et al. 2010). It reaches up to three- to fourfold at 48 h of desiccation, as measured by the decay of the luminous substance MPEC in the presence of the hypoxanthine–xanthine oxidase system as the source of ROS (Gusev et al. 2010). Similarly, *Belgica antarctica*, which often encounters desiccation, was also shown to have a high antioxidant capacity (Grubor-Lajsic et al. 1996), due to constantly overexpressed SOD (Lopez-Martinez et al. 2008).

As the elevated expression of antioxidant genes demonstrates, *P. vanderplanki* has a specific system for gene regulation under desiccation conditions. Putative molecular triggers for this regulation include a large variety of stressful chemical factors related to desiccation. For example, we can cite both intracellular and extracellular components, whose increase in concentration is tightly related to the increase of osmolarity and ionic strength. Among these components, extensively accumulated trehalose may play this trigger role. Since water loss is tightly related to the increase in ROS generation, this also can be a trigger for anhydrobiosis regulation. We should note that anhydrobiosis in *P. vanderplanki* is not regulated by the central nervous system, since larvae without their brains and related hormone-producing glands are still able to enter anhydrobiosis and even move after rehydration (Watanabe et al. 2002). Moreover, isolated fat body from *P. vanderplanki* larvae is still capable of accumulating trehalose and successfully tolerating desiccation (Watanabe et al. 2005).

The regulation of *P. vanderplanki*-specific genes that are probably related to desiccation tolerance is not restricted to the desiccation context only. For example, the expression of antioxidant genes varies considerably throughout different life cycle stages. Almost all antioxidant genes specific to *P. vanderplanki* show an expression

Table 14.2 Change in the expression of antioxidant genes in *P. vanderplanki* between larvae in controls and following 24 h of desiccation (Gusev et al. 2014). Green color highlights greater than twofold changes in expression

Common insect genes		P. vanderplanki-specific genes	
Gene names	Fold change at 24 h desiccation	Gene names	Fold change at 24 h desiccation
Cat	2.5	*Gtpx1*	1.4
CCS	1.1	*Sod4, Zn/Cu*	8.4
Grx1	6.7	*Sod5, Zn/Cu*	2.7
Grx1	2.2	*TRX genes*	779.6
Grx2	2.1		259.5
Grx-like1	3.9		100.0
MsrA	1.4		43.1
MsrB	0.9		35.2
Rsod	0.4		34.3
Sod1, Zn/Cu	0.6		13.6
Sod2, Mn	0.8		10.3
Sod3, Zn/Cu	1.0		10.0
Sodq	0.5		8.6
Tpx1	0.8		8.3
Tpx2	0.2		7.8
Tpx3	0.6		7.3
Tpx4	0.1		7.2
Tpx5	0.7		6.6
Trx/Gtx	1.7		6.2
Trx-1	1.7		5.2
Trx-1	1.0		3.3
Trx1-like1	1.5		3.2
Trx1-like2	0.7		1.8
Trx1-like3	1.1		1.5
Trx-2	1.7		
Trx-3	1.2		
Trxr-1	1.4		

peak at the larval stage, which is the only life stage that can survive desiccation (Table 14.3). In contrast, almost all common insect antioxidant genes in the *P. vanderplanki* genome have highest expression during the egg stage (Table 14.3). Nevertheless, they retain sufficient expression (more than 0.5 of the maximal one) at the next larval stage.

Moreover, in addition to the stage-dependent expression of antioxidant system genes, there is an obvious requirement for their tissue- and organ-specific regulation. For example, the fat body of *P. vanderplanki* larvae performs extensive synthesis of trehalose until very late stages of dehydration (Watanabe et al. 2005). Thus, if trehalose is really one of the triggers for the expression of some anhydrobiosis-related genes, as speculated before, the fat body should achieve anhydrobiosis preparation earlier, compared to other tissues. In addition, we have shown that

Table 14.3 Changes in the expression of antioxidant genes in *P. vanderplanki* between larvae and other stages of development (Gusev et al. 2014). The colors reflect a change in expression, with bright red depicting the highest expression level among different lifecycle stages

Common insect genes					P. vanderplanki-specific genes				
Gene	Expression, fold change				Gene	Expression, fold change			
	Egg	Larva	Pupa	Adult		Egg	Larva	Pupa	Adult
Cat	0.3	1.0	0.1	0.3	*Gtpx1*	0.2	1.0	0.0	0.1
CCS	1.1	1.0	0.4	0.8	*Sod4, Zn/Cu*	0.0	1.0	0.0	0.1
Grx1	10.5	1.0	3.3	2.7	*Sod5, Zn/Cu*	0.0	1.0	0.0	0.1
Grx1	4.3	1.0	2.6	1.6	*TRX genes*	0.6	1.0	0.6	0.1
Grx2	5.2	1.0	3.4	2.1		2.1	1.0	0.9	2.2
Grx-like1	1.4	1.0	1.2	0.1		0.1	1.0	0.0	0.0
MsrA	1.0	1.0	1.7	0.9		0.8	1.0	0.3	0.0
MsrB	3.1	1.0	1.1	1.4		0.0	1.0	0.0	0.1
Rsod	3.5	1.0	4.2	1.0		1.3	1.0	0.6	18.4
Sod1 (Zn/Cu SOD)	1.7	1.0	0.8	0.9		0.4	1.0	0.1	0.1
Sod2 (MnSOD)	1.3	1.0	1.1	0.7		-0.1	1.0	0.0	-0.6
Sod3 (Zn/Cu SOD)	0.1	1.0	0.1	0.2		0.3	1.0	0.1	1.1
Sodq	0.3	1.0	0.5	8.2		0.0	1.0	0.0	0.0
Tpx1	1.2	1.0	0.5	0.6		0.2	1.0	0.0	0.1
Tpx2	1.5	1.0	0.3	0.5		0.1	1.0	0.0	0.1
Tpx3	1.5	1.0	0.6	0.7		0.0	1.0	0.0	0.0
Tpx4	0.3	1.0	0.3	0.6		0.0	1.0	0.0	0.0
Tpx5	3.2	1.0	0.9	1.8		0.4	1.0	0.1	0.1
Trx/Gtx	2.5	1.0	1.0	1.8		0.1	1.0	0.0	0.1
Trx-1	2.1	1.0	2.9	4.1		0.1	1.0	0.0	0.0
Trx-1	1.3	1.0	0.3	0.7		0.0	1.0	0.0	0.0
Trx1-like1	4.1	1.0	2.1	2.1		0.0	1.0	0.0	0.1
Trx1-like2	2.5	1.0	3.2	2.0		0.8	1.0	0.0	0.2
Trx1-like3	1.6	1.0	1.5	2.5		0.0	1.0	0.2	0.6
Trx-2	2.5	1.0	0.8	1.0					
Trx-3	2.2	1.0	1.8	1.5					
Trxr-1	1.3	1.0	1.5	2.4					

some cells in dorsal vessels and nerves still remain apparently alive after fast desiccation and rehydration, which were sufficient to kill the whole larva and most of its cells (Nakahara et al. 2010). This observation suggests that different tissues show different levels of desiccation tolerance in *P. vanderplanki*. Such an intrinsic variation of desiccation tolerance among cells and tissues supports the hypothesis of a difference in the basal expression of antioxidant genes.

14.4 Thioredoxin (TRX) Subsystem

As mentioned above, a remarkable feature of the *P. vanderplanki* antioxidant system is the expansion of the TRX gene set (Table 14.1). TRX are small redox proteins existing in all live organisms, which perform regulation of the protein dithiol/disulfide balance that is tightly related to antioxidant defense and redox signaling (Meyer et al. 2008). The TRX system also participates in direct ROS scavenging, providing electrons to peroxiredoxins (a type of thiol-dependent peroxidase).

In addition to the three TRX genes that are conserved in insects, including *P. nubifer* (Corona and Robinson 2006; Gusev et al. 2014), the *P. vanderplanki* genome contains a second copy of the TRX1 gene and 21 additional TRX paralogs. These additional paralogs are organized in two ARIds and are unlinked to the TRX set conserved in other insects (Gusev et al. 2014). *P. vanderplanki*-specific TRX genes are the most strongly upregulated antioxidant genes in desiccating larvae, whereas the response of typical conserved insect TRX genes is moderate. Following the role of mitochondria as one of the major sources of ROS, some *P. vanderplanki* TRX contain a mitochondrial targeting sequence, as predicted by both TargetP (http://www.cbs.dtu.dk/services/TargetP/) (Emanuelsson et al. 2000) and iPSORT servers (http://ipsort.hgc.jp/) (Bannai et al. 2002). Mitochondrial localization is predicted for the common insect TRX-1, similarly to TRX-1 of *P. nubifer* or TRX-2 of *D. melanogaster* (Patenaude et al. 2004). In addition, mitochondrial targeting of PvTRX6, which is highly expressed during desiccation, is complemented by the predicted colocalization of the genes for thioredoxin reductase (TrxR) protein (Pv.12829), and one of the *P. vanderplanki* peroxiredoxins (Pv.03504). Thus, *P. vanderplanki* is likely to have a TRX-dependent system of antioxidant defense in mitochondria that is supplemented by desiccation-dependent overexpression of mitochondria-targeted TRX-6. Besides an antioxidant function, upregulation of TRX also may be related to their role in DNA repair and synthesis of deoxyribonucleotides (Holmgren and Sengupta 2010), since desiccation is known to cause severe DNA damage (Gusev et al. 2010). The presence of functions different from antioxidant defense for these *P. vanderplanki*-specific TRX proteins is supposed by the absence of upregulation of thioredoxin peroxidases (TPx), which are necessary to link TRX to ROS scavenging (Table 14.2). The gene for TrxR, which is important for TRX function in ROS neutralization, is also only slightly upregulated by desiccation (Table 14.2).

14.5 Glutathione Peroxidase (GPx)

Glutathione and enzymes dependent on it are important components of cell defenses against ROS and toxic agents (Foyer and Noctor 2011). Members of the GPx family reduce free hydrogen peroxide (Pompella et al. 2003) and peroxidized lipids (França et al. 2007), thereby scavenging ROS and protecting biological membranes. GPx is the most abundant transcript of any antioxidant gene in the larvae, both in normal conditions and after 24 h of desiccation (Gusev et al. 2014). Based on the expressed sequence tag database and mRNA-seq data (Cornette et al. 2010; Gusev et al. 2014), we can conclude that *P. vanderplanki* GPx is expressed in four splice forms (Gusev et al. 2014). Two GPx forms that

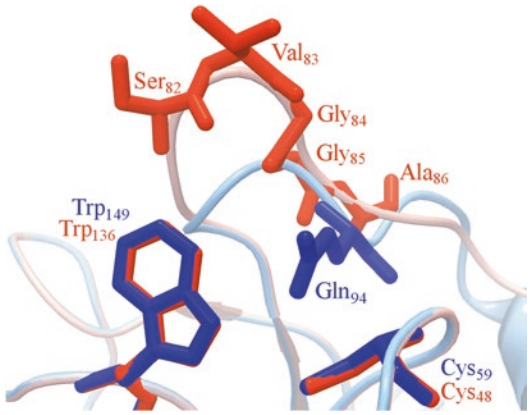

Fig. 14.2 Structural alignment of the *P. vanderplanki* GPx protein model missing the catalytic glutamine residue (splice variant D, in red) and human glutathione peroxidase 4 (in blue; PDB entry 2OBI.A Scheerer et al. 2007). The model of *P. vanderplanki* glutathione peroxidase structure was built with the SWISS-MODEL server (Biasini et al. 2014) using 2OBI.A structure as a template. Visualization was done using CLC Genomics Workbench 8.0. Catalytic residues of human glutathione peroxidase 4 and corresponding residues of *P. vanderplanki* GPx are depicted on the figure

are overexpressed in desiccation do not contain a catalytic glutamine residue at a position typical for other known GPx (Maiorino et al. 2007). Structural modeling using human GPx as a template (PDB ID 2obi.A; Scheerer et al. 2007) revealed that the missing glutamine of the catalytic triad is not substituted by an adjacent residue (Fig. 14.2). Interestingly, these GPx forms become more represented during desiccation, composing more than half of all GPx mRNA after 24 h of desiccation. This substitution reflects a change in protein specificity or function. In this context, it is important that most GPx enzymes accept TRX proteins as a reducing substrate (Maiorino et al. 2007). *P. vanderplanki*-specific splice forms of GPx share peculiar features of TRX-specific GPx, such as a cysteine residue within the fourth helix and an absence of the subunit interfaces of tetrameric GPx enzymes (Maiorino et al. 2007). These data seem to be related to the overexpression of TRX genes in *P. vanderplanki* during desiccation and the presence of previously unidentified variations of catalytic residues in both *P. vanderplanki* TRX proteins and GPX.

14.6 Superoxide Dismutases (SOD)

SOD are important antioxidant enzymes that perform detoxification of the superoxide anion into hydrogen peroxide, which in turn is reduced to water by catalase (Zelko et al. 2002). *P. vanderplanki* expresses two cytoplasmic and one mitochondrial SOD, which are conserved in other insects, including *P. nubifer*. The *P. vanderplanki* genome also contains two additional SOD-encoding genes, SOD4 and SOD5. These genes are located separately from other SOD genes and become upregulated after 24 h of desiccation (Table 14.2). At this desiccation stage, they represent around half of all newly synthesized SOD mRNAs. Expression of common insect SOD genes in *P. vanderplanki* is not increased in the desiccation process (Table 14.2). Upregulation of *P. vanderplanki*-specific SOD genes highlights their importance for desiccation tolerance. Furthermore, according to MidgeBase (http://bertone.nises-f.affrc.go.jp/midgebase/), SOD4 and SOD5 genes are expressed in *P. vanderplanki* almost solely at the larval stage, the only stage at which this insect can survive desiccation. Remarkably, primary sequence analysis suggests that the SOD4 and SOD5 genes are not paralogs of typical insect SOD genes.

Among other SOD enzymes, manganese-containing SOD2 is remarkable due to its well-known function in mitochondria, which are usually considered as the main source of ROS inside the cell (Indo et al. 2007). However, the SOD2 gene of *P. vanderplanki* is not upregulated in response to desiccation (Table 14.2), while the upregulated SOD4 and SOD5 genes encode Cu, Zn-SOD that are typically considered as cytoplasmic or extracellular enzymes. SOD4 and SOD5 proteins of *P. vanderplanki* do not contain targeting sequences, as predicted by the subcellular protein localization prediction programs TargetP (Emanuelsson et al. 2000), SignalP (http://www.cbs.dtu.dk/services/SignalP-2.0/) (Petersen et al. 2011) and iPSORT (Bannai et al. 2002). However, human Cu, Zn-SOD has been shown to translocate into the intermembrane

space of mitochondria regardless of target sequences (Weisiger and Fridovich 1973).

In general, Cu, Zn-SOD are partially dependent on copper chaperone protein (CCS), which mediates insertion of the essential copper cofactor and formation of the intramolecular disulfide bond (Brown et al. 2004; Furukawa et al. 2004). The extent of this dependency is variable: yeast SOD is inactive without CCS (Carroll et al. 2004), but SOD of *Caenorhabditis elegans* and *Megavirus chilensis* have been shown to be totally independent of it (Jensen and Culotta 2005; Lartigue et al. 2015). The dependence of SOD on CCS is linked to the presence of two proline residues near the C-terminus (Carroll et al. 2004), whereas the molecular features allowing SOD to be independent of CCS are still unclear (Leitch et al. 2009). In this context, it is remarkable that the CCS expression level in *P. vanderplanki* is similar to that in *P. nubifer*, and it does not change in desiccation (Table 14.2) despite the huge increase of Cu, Zn-SOD expression. Thus, we assume that *P. vanderplanki* SOD4 and SOD5 are at least partially independent of CCS.

14.7 Nonprotein Antioxidants in *P. vanderplanki*: Trehalose as a Potential Antioxidant Agent

Outside the protein-based pathways of antioxidant defense, molecular antioxidants, such as glutathione and ascorbate, can also directly scavenge ROS (França et al. 2007). However, the role of these antioxidants in *P. vanderplanki* antioxidant defense seems to be secondary or even negligible. The *P. vanderplanki* genome lacks L-gulonolactone oxidase, an enzyme that performs the last step of ascorbate biosynthesis. Ascorbate peroxidase, an enzyme facilitating ascorbate-dependent ROS scavenging, is also absent. Food as possible ascorbate sources can be neglected, because *P. vanderplanki* reared continuously on milk agar without ascorbate addition has shown successful desiccation survival (Watanabe et al. 2005). Similarly, low expression of enzymes performing glutathione synthesis is found in *P. vanderplanki*. Genes of glutathione synthetase and gamma-glutamylcysteine synthetase described in *Chironomus riparius* (Nair et al. 2013) have direct orthologs in *P. vanderplanki*, but these are expressed at low levels in all life stages. These data do not contradict the elevated expression of GPx because, in *Drosophila,* the reduction of Cys residue in the catalytic triad of this enzyme is maintained by TRX as a reducing agent instead of glutathione (Maiorino et al. 2007). If so in *P. vanderplanki*, high expression of previously unknown forms of GPx seems to be related to the high expression of TRX which include also previously undescribed forms.

However, besides ascorbate and glutathione, trehalose may play a role as a nonprotein antioxidant in *P. vanderplanki.* Trehalose is a nonreducing sugar that accumulates extensively in *P. vanderplanki* larvae during desiccation, reaching up to 20% of the dry mass (Sakurai et al. 2008; Watanabe et al. 2002). In desiccated cells, trehalose replaces the normal intracellular medium with a very stable biological glass, thereby stabilizing the structure of biological membranes and proteins (Crowe 2007; Sakurai et al. 2008). The superior protective properties of trehalose in comparison to other sugars have led to its widespread use as a chemical chaperone and protectant (reviewed in Crowe 2007). A growing number of reports state that trehalose possesses a significant antioxidant effect in dried and non-dried cells. It significantly reduces oxidative damage of *Saccharomyces cerevisiae* cells and plants during desiccation or exposure to heat, osmotic shock, or oxidative agents (Benaroudj et al. 2001; Pereira Ede et al. 2003; Herdeiro et al. 2006; da Costa Morato Nery et al. 2008). Trehalose accumulation may inhibit damage of cell components in the dry state by nonenzymatic reactions (França et al. 2007), which otherwise would cause a loss of cell viability in the dry state (Sun and Leopold 1995; Buitink and Leprince 2004). However, trehalose itself is not a strong molecular antioxidant, because it is a nonreducing sugar with an absence of strong nucleophilic groups in its molecule. Its antioxidant effects are linked to the ability to interact with proteins and membranes and thereby form a chemical molecu-

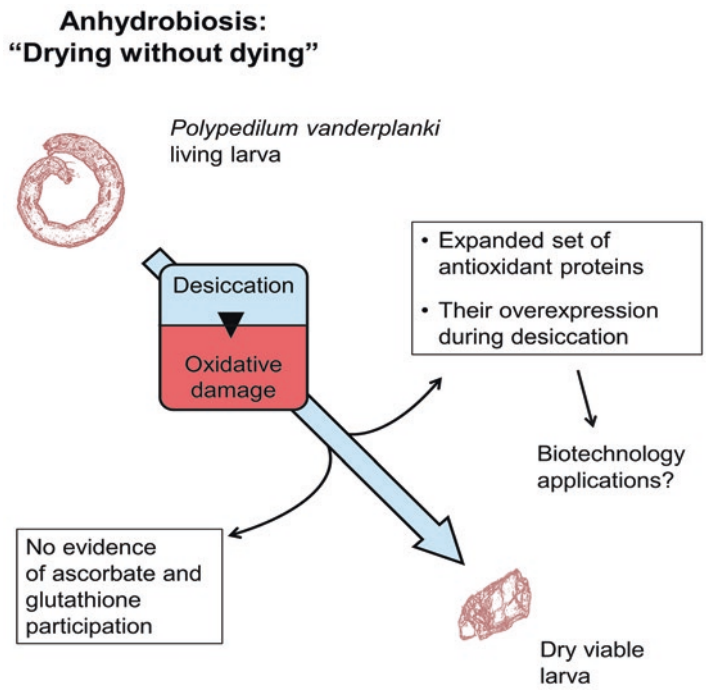

Fig. 14.3 Graphical schema for the antioxidant system during induction of anhydrobiosis in *P. vanderplanki* larva. The larva can survive even after losing its body water almost completely. This ability is tightly related to the capability to overcome extraordinary oxidative stress caused by water loss. The features of *P. vanderplanki* antioxidant system include an expansion of antioxidant genes set and induction of these genes during desiccation. Enzymes required for the synthesis of ascorbate and glutathione and their antioxidant function are low expressed or absent, suggesting secondary role of these antioxidant molecules. The antioxidant system of *P. vanderplanki* could be used in the development of technologies for the dry preservation of biomaterials, being efficient in the specific context under desiccation conditions

lar chaperone shield (Oku et al. 2003; Crowe 2007; França et al. 2007).

14.8 Conclusion

The anhydrobiotic insect *P. vanderplanki* has evolved a powerful antioxidant system mandatory for its survival after desiccation, which is tightly related to high oxidative stress. On a genomic level, this is reflected by an unprecedented expansion of the set of antioxidant genes, as well as the specific regulatory elements performing the upregulation of antioxidant genes in response to desiccation (Fig. 14.3). Due to the high expression of antioxidant genes during desiccation, the antioxidant system of *P. vanderplanki*, at least its anhydrobiosis-involved component, is likely to be based on antioxidant proteins. Molecular antioxidants, such as ascorbate and glutathione, seem to be secondary in the *P. vanderplanki* antioxidant system, because enzymes necessary for their synthesis and antioxidant function are absent or low expressed. A growing body of evidence suggests that trehalose, which accumulates at high levels in desiccated *P. vanderplanki* larvae, could be an important player in the antioxidant system of *P. vanderplanki*. Since anhydrobiosis is proposed as a model for biomaterial preservation, related oxidative damage should be taken into account. The antioxidant system of *P. vanderplanki* could be used as a source of protective proteins that work well in the specific context of desiccation conditions.

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15 Physicochemical Aspects of the Biological Functions of Trehalose and Group 3 LEA Proteins as Desiccation Protectants

Takao Furuki and Minoru Sakurai

Abstract

In this review, we first focus on the mechanism by which the larva of the sleeping chironomid, *Polypedilum vanderplanki*, survives an extremely dehydrated state and describe how trehalose and probably late embryogenesis abundant (LEA) proteins work as desiccation protectants. Second, we summarize the solid-state and solution properties of trehalose and discuss why trehalose works better than other disaccharides as a desiccation protectant. Third, we describe the structure and function of two model peptides based on group 3 LEA proteins after a short introduction of native LEA proteins themselves. Finally, we present our conclusions and a perspective on the application of trehalose and LEA model peptides to the long-term storage of biological materials.

Keywords

Anhydrobiosis · Vitrification · Water replacement model · Entropy · Disaccharide · Late embryogenesis abundant protein · LEA peptide · Sleeping chironomid

Abbreviations

BDG	β-D-galactosidase
G3LEA	Group 3 late embryogenesis abundant
IDP	Intrinsically disordered protein
LDH	Lactate dehydrogenase
MD	Molecular dynamics
POPC	1-palmitoyl 2-oleoyl-sn-glycero-3-phosphatidylcholine

15.1 Introduction

Some organisms can survive severe drought in their environment using a strategy called anhydrobiosis (Crowe et al. 1992; Clegg 2001). During anhydrobiosis, such organisms lose almost all their body water and appear to be inanimate (i.e., dead), because no sign of life can be detected, but they are actually in a state of suspended animation. They differ from inanimate material in that they are capable of revival after rehydration. How should we distinguish between these three states (living state, death, and anhydrobiosis)?

According to Schrödinger (1967), a living organism feeds upon negative entropy (i.e., other organisms or ordered systems) to compensate the entropy increase it produces by being alive and thereby to maintain itself on a stationary and fairly low entropy level. This allows living sys-

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tems to be highly ordered and yet not to disobey the second law of thermodynamics, which states that the entropy of an isolated system increases over time and approaches an inert state of maximum entropy. An organism in the anhydrobiotic state cannot assimilate negative entropy from its environment because it is in a state of suspended metabolism, but it must nevertheless minimize any entropy increase due to bodily decay. Therefore, the anhydrobiotic state is different from both the living and death states.

How do the anhydrobiotic organisms maintain their ordered state? There should be an "entropy barrier" by which the order-to-disorder process is significantly retarded: it corresponds to a kinetic barrier, which slows down, but does not abrogate, entropic processes. Desiccation protectants are known to accumulate in diverse anhydrobiotic organisms and are thought to work as an entropy barrier. The nonreducing disaccharides, trehalose (animals, fungi) and sucrose (plants), are representative desiccation protectants (Crowe et al. 1992; Crowe 2002; Oliver et al. 2002). However, some desiccation-tolerant animals such as bdelloid rotifers and tardigrades do not require or even appear to make trehalose (Lapinski and Tunnacliffe 2003; Hengherr et al. 2008), which implies that other molecules are likely to contribute significantly to desiccation tolerance. With respect to protective proteins, the late embryogenesis abundant (LEA) proteins are the best-characterized examples. LEA proteins were initially discovered more than three decades ago in cotton seeds (Dure et al. 1981). Later, they were also found in non-plant organisms (Tunnacliffe and Wise 2007; Shih et al. 2008; Hand et al. 2011).

How do these protectants work as an entropy barrier? We first take as an example of a well-studied anhydrobiotic organism the so-called sleeping chironomid, *Polypedilum vanderplanki*; the larvae of this midge can survive extreme dehydration, and this is associated with the accumulation of trehalose and LEA proteins (Watanabe et al. 2003; Kikawada et al. 2006). Second, we discuss why trehalose is superior to other disaccharides as a desiccation protectant. Third, we discuss the mechanisms of LEA protein function based on our recent studies of two peptides modeled on group 3 LEA (G3LEA) proteins. Finally, we present our conclusions and a perspective on the use of trehalose and G3LEA model peptides for the long-term storage of biological materials. This chapter is not intended to be a comprehensive review of the literature on trehalose and LEA proteins: for more extensive reviews, please see (Tunnacliffe and Wise 2007; Shih et al. 2008; Jain and Roy 2009; Sakurai 2009; Tunnacliffe et al. 2010; Ohtake and Wang 2011; Hand et al. 2011).

15.2 Mechanism of Desiccation Tolerance in the Sleeping Chironomid

The larvae of *P. vanderplanki* dwell in temporary rock pools in semiarid regions of Africa. In the dry season, the larvae become severely desiccated but are able to recover after rehydration when the next rain arrives. Okuda's group at NIAS in Japan succeeded in inducing *P. vanderplanki* larvae to enter anhydrobiosis under laboratory conditions (Watanabe et al. 2002). When the larvae are slowly dehydrated over 72 h, they accumulate a large amount of trehalose (36 μg/individual) and successfully enter anhydrobiosis. However, when dehydrated quickly over a few hours, the larvae produce relatively little trehalose (2 μg/individual), resulting in the failure of anhydrobiosis. In both slowly and rapidly dried larvae, there is no apparent difference between the amounts of total protein, triacylglycerol, and water (≈3% wt./dry individual). These observations suggest that trehalose is responsible for the induction of anhydrobiosis (Sakurai et al. 2008). Trehalose has an α,α-1,1 glycosidic linkage (Fig. 15.1a), which is unique to this sugar among naturally occurring gluco-disaccharides, i.e., disaccharides composed of two glucose units, and thus exhibits a unique vibration band at 992 cm^{-1}. Indeed, by FTIR spectroscopy, a clear peak can be observed at this position for a slowly dehydrated larva, whereas such a peak is not detected when larvae are rapidly dehydrated. The intensity distribution of this peak in slowly dehydrated larvae clearly indicates that trehalose is almost uniformly distributed through the larval body (Fig. 15.2a, b) (Sakurai et al. 2008). As

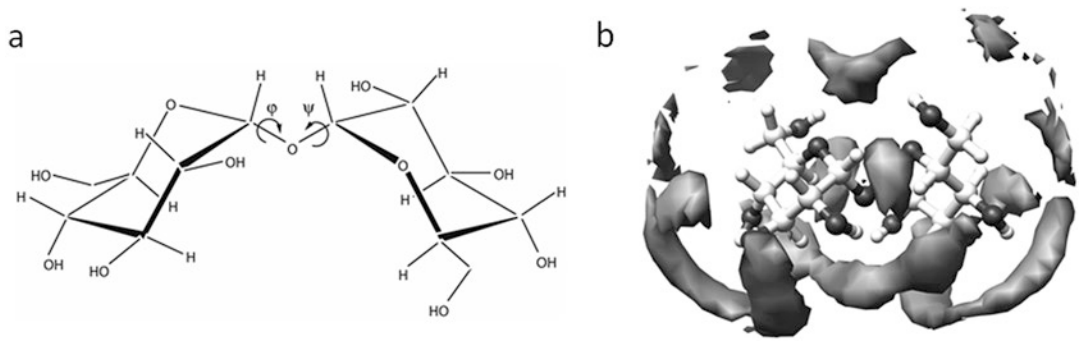

Fig. 15.1 (**a**) Chemical structure of trehalose and (**b**) its hydration structure. Cloud-like regions represent the iso-probability surface of water oxygen atoms

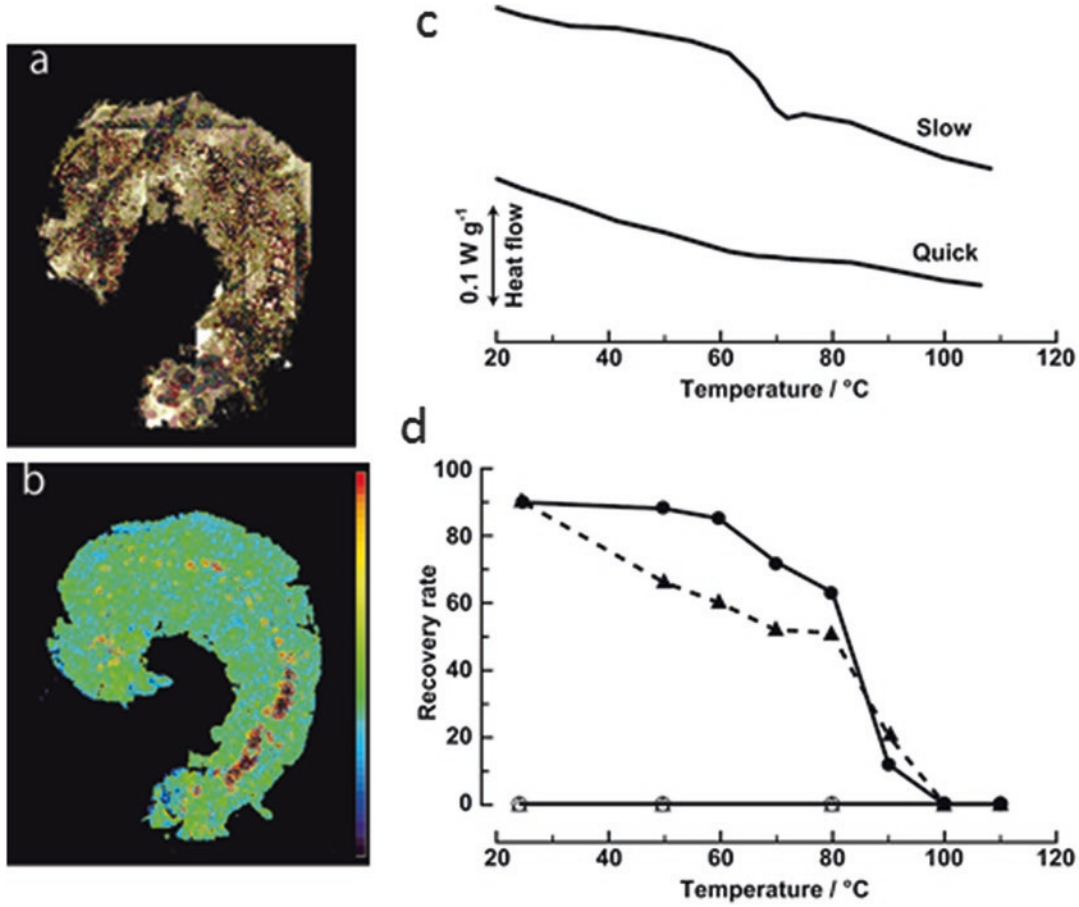

Fig. 15.2 (**a**) Optical and (**b**) FTIR imaging of a slowly dehydrated larvae. (**c**) DSC thermograms for slowly and rapidly dehydrated larvae. Warm colors indicate higher intensity—i.e., larger amounts of the molecule. (**d**) Dependence of the recovery rate after rehydration on exposure to high temperatures in slowly (filled symbols) and rapidly (open symbols) dehydrated larvae. Circles and triangles show recovery after exposure to high temperature for 5 min and 1 h, respectively. (Data from Sakurai et al. 2008 (Copyright (2008) National Academy of Sciences, U.S.A))

shown in Fig. 15.2c (Sakurai et al. 2008), the DSC thermogram for slowly dehydrated larvae exhibits a clear baseline shift in a step-wise manner, a feature of glass-rubber transition, suggesting that anhydrobiotic larvae are in the glassy state at ambient temperatures. The onset, middle, and end temperatures of the transition are 62 °C, 65 °C (the glass transition temperature, T_g), and 71 °C, respectively, in the representative experiment shown. In contrast, a thermogram of rapidly dehydrated samples exhibits none of these features.

Anhydrobiotic larvae are remarkably stable at elevated temperatures, providing these temperatures do not approach the T_g. Figure 15.2d shows the recovery rate after rehydration following exposure of dried larvae to different temperatures for 5 min or 1 h (Sakurai et al. 2008). For the slowly dehydrated larvae, a high recovery rate of 60–90% is typical for temperatures up to 50 °C, although the longer exposure time results in a slightly lower survival rate. Exposure to higher temperatures gradually decreases the recovery rate, and no survival occurs beyond ca. 100 °C. In marked contrast, the rapidly dehydrated larvae never recover after incubation at any temperature tested. These observations suggest that dried larvae revive only when sufficient trehalose has accumulated prior to full desiccation and when this trehalose vitrifies and is maintained in the glassy state, i.e., below the T_g.

In such a trehalose-associated glassy state, cellular components would be incorporated in a rigid sugar glass and thus have extremely limited mobility. In other words, the trehalose sugar glass functions as an entropy barrier. As a result, although an anhydrobiotic larva appears to be an amorphous, inert solid, its biological components maintain the spatial arrangements required for normal physiology. Therefore, the larvae can return to the living state by absorbing water. This scenario is consistent with the so-called vitrification hypothesis (Crowe et al. 1998).

Together with vitrification, the water replacement and water entrapment mechanisms might also operate in the dry state; these mechanisms are not mutually exclusive. The former suggests that sugars can replace water molecules by forming hydrogen bonds with polar residues of lipid and/or protein molecules (Crowe et al. 1992, 1998; Crowe 2002). The latter indicates that sugars concentrate water near the surfaces of membrane and protein, and thus bound water is not lost, even on dehydration (Belton and Gil 1994). FTIR spectra show that the P=O asymmetric stretch vibration assigned to the head groups of phospholipids shifts to the lower wavenumber side more in slowly than in rapidly dehydrated larvae (Sakurai et al. 2008). This suggests that, in the former sample, hydrogen bonds are formed between the polar head groups of phospholipids and (probably) trehalose, consistent with the water replacement hypothesis. In addition, the temperature dependence of the symmetric CH_2 stretch vibration of fatty acid chains shows that the gel-to-liquid crystalline transition temperature of the membrane is significantly lowered in slowly dehydrated larvae, i.e., cellular membranes in this sample are likely to be in the liquid crystalline state at room temperature in spite of the absence of water (Sakurai et al. 2008). Thus, an unfavorable phase transition is avoided during the subsequent rehydration process, a key factor that allows cellular membranes to retain their integrity and successfully recover from desiccation.

On the basis of the above results, trehalose is thought to be a major player in inducing and maintaining the anhydrobiosis of *P. vanderplanki* larvae. However, it can be inferred that there is also another contributor to the stabilization of the vitreous state in the larval body. This is partly due to the fact that the glass transition temperature of slowly dehydrated larvae shifts less with increasing water content than expected from theoretical values calculated for a binary mixture of pure trehalose and water (Sakurai et al. 2008). Possible candidates for these additional stabilizing molecules are the LEA proteins, which have been suggested to reinforce biological glasses (Wolkers et al. 2001). LEA proteins were first reported in *P. vanderplanki* by Kikawada et al. (2006), and currently 27 kinds of LEA protein are known to occur in the sleeping chironomid (Gusev et al. 2014; Hatanaka et al. 2015). The details of the

structure and function of LEA proteins will be discussed in Sect. 15.4.

15.3 Physicochemical Properties of Trehalose

In this section, we discuss why trehalose is a good desiccation protectant. Trehalose has only a single energy minimum around the glycosidic bond: the minimum is located at the glycosidic dihedral angles of (ϕ, φ)=(−60°, −60°), corresponding to the gauche conformation (Dowd et al. 1992). The energy conformation minimum is similar to a clamshell (Fig. 15.1). Other types of glycosidic linkage, including (1–4) and (1–6) bonds, allow for multiple conformers (Perić-Hassler et al. 2010). Therefore, the less flexible α,α-1,1-glycosidic linkage is responsible for many of the unique properties of trehalose in the solid and solution states. In addition, the glycosidic bond of trehalose is chemically very stable, rendering the disaccharide less susceptible to hydrolysis into two glucose molecules (Ohtake and Wang 2011).

15.3.1 Solid-State Properties of Trehalose

15.3.1.1 Glassy State

Table 15.1 lists the glass transition temperatures for all of the naturally occurring gluco-disaccharides and for sucrose (Oku et al. 2004). The T_g (115 ± 2 °C) of trehalose is the highest among the gluco-disaccharides, although the value is not particularly remarkable, or at least is not anomalous. The activation energy of the translational diffusion of molecules forming the glass of interest, ΔE_{relax}, is a direct measure of the physical stability of the vitrified matrix. As shown in Table 15.1, the ΔE_{relax} of trehalose is larger than those of the other gluco-disaccharides and sucrose by >150 kJ mol^{-1}. Based on these data and the Arrhenius equation, the degradation rate of a trehalose glass is estimated to be four to seven times slower than those of a maltose glass or a sucrose glass, which suggests that vitrified trehalose is more stable and hence should represent a better entropy barrier.

Table 15.1 Glass transition temperatures (T_g) and activation energies of enthalpy relaxation (ΔE_{relax}) of dry amorphous disaccharides

Sugar	Tg (°C)	ΔE_{relax} (kJ mol^{-1})
Trehalose	116	401.0
Neotrehalose	105	223.4
Kojibiose	118.3	273.1
Sophorose	88.6	283.3
Nigerose	81.1	270.5
Laminaribiose	106.7	314.1
Maltose	84.5	292.4
Isomaltose	89.5	279.9
Cellobiose	100.2	307.4
Gentiobiose	94.4	284.1

The origin of the excellent stability of trehalose glass was revealed by a comparative study between trehalose and neotrehalose (α-D-glucopyranosyl β-D-glucopyranoside) using molecular dynamics (MD) simulations (Kawasaki et al. 2006). The simulations successfully reproduced the difference in glass transition temperatures between these sugars: the calculated T_g of trehalose is 10 °C higher than that of neotrehalose. Despite detailed analysis of the hydrogen bond networks in their glassy matrices, there are no obvious differences that might account for the disparity in their T_g values. Analysis of the distribution of free volumes in the glassy matrix, however, suggests that the void size in a trehalose glass is almost uniformly distributed, while in a neotrehalose glass, it has a more diverse distribution. It is likely that the conformational unity (rigidity) of trehalose, originating from the α,α-1,1-glycosidic linkage, contributes to the formation of a more uniform amorphous solid, probably resulting in a more stable glass.

Generally, water is a good plasticizer of glassy matrices: on absorption of water from the environment, the glass transition temperature is lowered, which may provide irreparable damage to biomaterials stored in organic glasses. The properties of trehalose should minimize this risk of devitrification. This sugar can be crystallized as hydrous forms from the amorphous state, leading to a decrease in the residual water content of the

remaining amorphous matrix (Aldous et al. 1995). As a result, the glass transition temperature T_g becomes higher, or at least the depression of T_g caused by plasticization through water uptake is more or less avoidable (Aldous et al. 1995; Crowe et al. 1996).

15.3.1.2 Polymorphism

Trehalose crystallizes in the dihydrate form (usually referred to as Th or form I) from its aqueous solution. The thermodynamic quantities (heat capacity, enthalpy, entropy, and free energy) of the dihydrate are measured as a function of temperature in the range 13–300 K (Furuki et al. 2006). The two crystalline water molecules are easily activated on heating. Their bending vibration band at 1680 cm^{-1} shifts steeply to 1640 cm^{-1} at around 70 °C, which suggests that they convert from an icelike structure to a liquid-like one before melting (90 °C) of the crystal (Ako et al. 1998). The labile nature of this crystalline water causes solid-state trehalose to exhibit a unique polymorphism depending on atmospheric pressure (Furuki et al. 2008) or humidity (Furuki et al. 2005). Figure 15.3 shows the de- and rehydration behavior of Th under controlled humidity atmospheres (Furuki et al. 2005), where there are three different crystal forms: dihydrate Th; the anhydrous form, referred to as Tα (or form II); and another anhydrous form, referred to as Tβ (or form III). Under dry atmospheres, Tα is formed at 105 °C on dehydration of Th. It is highly hygroscopic and can be readily rehydrated back to Th when exposed to even low-humidity atmospheres. Tα crystals likely function as a water sink, reducing the risk of devitrification (Kilburn et al. 2006). Indeed, if a mixture of Tα and amorphous trehalose is exposed to moisture, water is absorbed more rapidly by the transformation from Tα to Th than by water absorption to amorphous trehalose (Nagase et al. 2002). This characteristic property of Tα can be understood from its crystal structure as follows: (i) the molecular arrangement in Tα is very similar to that of Th; (ii) there is a one-dimensional water channel throughout the crystal; and (iii) the intermolecular interactions between trehalose molecules in Tα are weaker than those in Tβ, which accounts for more rapid water uptake into the Tα crystal (Nagase et al. 2008).

15.3.2 Hydration Properties of Trehalose

In what situations do the water replacement and water entrapment mechanisms work well? Both mechanisms depend on the relative strength of the three pairs of interactions between protectant (sugar), target (protein or membrane), and water. Accordingly, the hydration of the sugar used is a key factor governing these mechanisms.

In Table 15.2, we compare the hydration numbers (n_h) of trehalose, maltose, and sucrose because these sugars have the same chemical formula ($C_{12}H_{22}O_{11}$) and mass (molecular weight

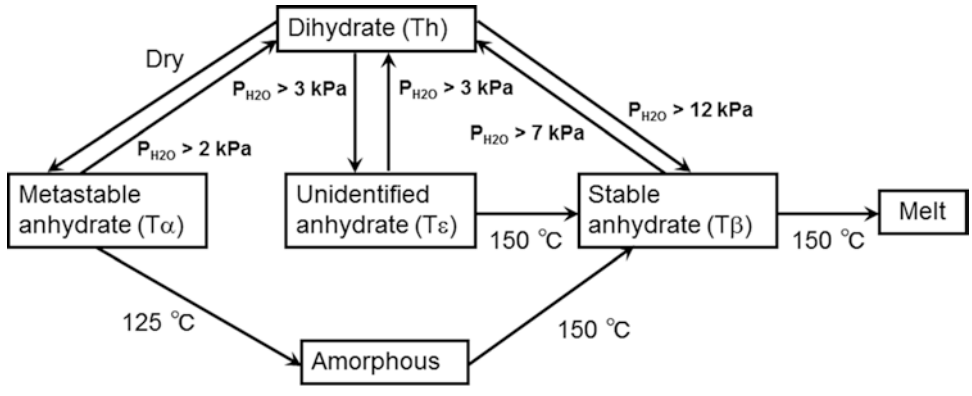

Fig. 15.3 Phase and state transitions of trehalose. P_{H2O} represents partial vapor pressure of water

Table 15.2 Comparison of hydration properties of disaccharides

Sugar	*e*-OH[a]	n_h					n_{DHN}[a]
		Viscosity[b]	Ultrasound[c]	QRNS[d]	DSC[e]	THz[f]	
Trehalose	8.0	8.0	15.3	9.0	8.0	39.8	48.3
Maltose	7.6	7.5	14.5	8.4	6.5	–	23.8
Sucrose	6.3	6.8	13.9	7.5	6.3	23.6	36.8

[a]Kawai et al. (1992)
[b]Obtained from viscosity measurements (Portmann and Birch 1995)
[c]Obtained from ultrasound measurements (Galema and Høiland 1991)
[d]Obtained from quasi-elastic neutron scattering measurements (Magazu et al. 2001)
[e]Obtained from DSC measurements (Kawai et al. 1992)
[f]Obtained from terahertz spectroscopic measurements (Shiraga et al. 2015)

342.3). For all three sugars, the n_h values from terahertz spectroscopy are larger than those obtained by other methods because water molecules in the second hydration shell are counted together with those in the first hydration shell. Regardless of the method used, trehalose has a larger hydration number. In addition, trehalose has a larger dynamic hydration number (n_{DHN}), which indicates that trehalose has a larger number of water molecules in the cybotactic region in which their rotational dynamics are slowed down by interaction with the solute. The n_h and n_{DHN} values correlate with the number of equatorial hydroxyl (*e*-OH) groups: the correlation originates from the fact that the spatial arrangement of the *e*-OH groups of the glucose ring shows a good match with that of the hexagonal icelike cluster in liquid water (Uedaira et al. 1989). The stronger hydration ability of trehalose is also supported by observations of the swelling behavior of hydrogel in sugar solutions: the equilibrium swelling ratio decreases in the order sucrose > maltose > trehalose (Furuki et al. 2009), which means that the polymer-water affinity is weakened as the sugar-water interaction strengthens in the order shown.

Choi et al. (2006) performed systematic computational work for 13 different homodisaccharides with different glycosidic linkages and showed that trehalose has a much larger number (2.8) of long-lived hydrogen bonds with water compared with the other 12 sugars. One of the most important findings from MD simulations is that trehalose has a highly anisotropic hydration shell, as shown in Fig. 15.1b. Interestingly, the concave side of the clamshell is fully hydrated, while there are pockets having no first hydration shell on the convex side. Due to the conformational rigidity of the sugar, a stable hydrogen bond network is formed between the *e*-OH groups of the glucose ring and surrounding water molecules on the concave side. In contrast, on the convex side, hydrophobic molecules such as benzene can bind to the hydrophobic pockets without dehydration penalty (Sakakura et al. 2011).

Taken together, trehalose can be classified as a kosmotrope or water-structure maker: the interaction between trehalose and water is much stronger than the water-water interaction. Trehalose has a larger hydration number, thereby being able to deliver a larger number of hydrogen bonding sites to a target biomolecule in place of water. Its high hydration ability is of course a great advantage for the water entrapment mechanism as well.

15.4 Physicochemical Aspects of the Structure and Function of Group 3 LEA Proteins

LEA proteins are classified into several groups according to their gene expression pattern and amino acid sequence (Tunnacliffe and Wise 2007; Shih et al. 2008). In the subsequent sections, we focus on group 3 LEA (G3LEA) proteins because they form the largest group of LEA proteins and also the main group of LEA proteins found in non-plant organisms.

15.4.1 The Structure of Native Group 3 LEA Proteins

G3LEA proteins are characterized by several tandem repeats of a loosely conserved 11-mer motif in the primary sequence (Tunnacliffe and Wise 2007; Shih et al. 2008). For example, the consensus motif of the 11-mer units of plant G3LEA proteins can be written "ΦΦΩXΦΨΩΨΦXΩ," where Φ, Ω, and Ψ represent hydrophobic residues, negatively charged or amide residues, and positively charged residues, respectively, and X represents a non-conserved amino acid residue (Dure 1993). Accordingly, the 11-mer motifs are rich in polar residues, rendering G3LEA proteins very hydrophilic in nature (Tunnacliffe and Wise 2007).

When polypeptide chains composed of the abovementioned 11-mer units form α-helical structures, they are expected to have an amphiphilic character due to a hydrophobic stripe formed by the apolar residues at positions 1, 2, 5, and 9 and a wider hydrophilic stripe formed by the polar residues at positions 3, 6, 7, 8, and 11 (Fig. 15.4a). Early computer modeling studies predicted that such amphiphilic α-helices dimerize in a right-handed coiled-coil arrangement through the interactions of the hydrophobic stripes (Dure 1993). Later, the formation of α-helical (possibly coiled-coil-like) structures was observed in the dry state for various G3LEA proteins originating from a nematode (Goyal et al. 2003), pea mitochondria (Tolleter et al. 2010), *Typha latifolia* pollen (Wolkers et al. 2001), a bdelloid rotifer (Pouchkina-Stantcheva et al. 2007), and *P. vanderplanki* (Hatanaka et al. 2013). However, in contrast to many globular proteins, G3LEA proteins are disordered in solution. Thus, they are regarded as intrinsically disordered proteins (IDPs), which are known to comprise perhaps one-third of eukaryotic proteins and which often undergo a disorder-to-order transition as a requirement for biological function (Uversky et al. 2000).

15.4.2 The Function of Native Group 3 LEA Proteins

G3LEA proteins function as protectants for proteins and membranes subjected to desiccation stress (Tunnacliffe and Wise 2007; Hand et al. 2011; Hincha and Thalhammer 2012). In vitro, a G3LEA protein from a nematode (AavLEA1) suppresses desiccation-induced aggregation and inactivation of enzymes such as lactate dehydrogenase (LDH) and citrate synthase (CS) (Goyal et al. 2005). Similar results can be obtained with water-soluble proteomes (Chakrabortee et al. 2007). AavLEA1 is also able to inhibit the spontaneous aggregation of polyglutamine-containing proteins within cells (Chakrabortee et al. 2007; Liu et al. 2011). Protection against desiccation-induced damage of enzymes is commonly observed for other G3LEA proteins (Grelet et al. 2005; Hatanaka et al. 2013;

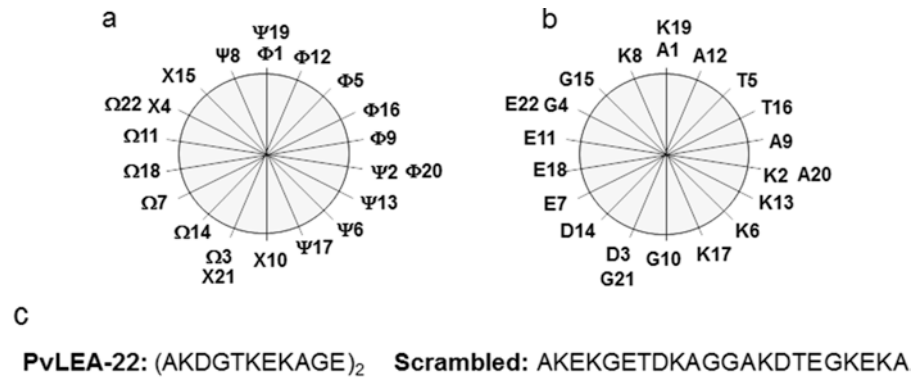

Fig. 15.4 α-helical wheel of (**a**) the consensus G3LEA protein 11-mer motif of plants and (**b**) the G3LEA model peptide, PvLEA-22; (**c**) the amino acid sequences of PvLEA-22 and the scrambled peptide

Boswell et al. 2014; Popova et al. 2015). It has been reported that some G3LEA proteins are also able to suppress the fusion of liposomes during desiccation (Tolleter et al. 2010; Moore et al. 2016) and others even to enhance desiccation tolerance in mammalian cells (Li et al. 2012).

To date, two hypotheses have been proposed as mechanisms by which G3LEA proteins function as desiccation protectants: (1) cytoskeleton formation (Wise and Tunnacliffe 2004) and (2) molecular shielding (Goyal et al. 2005; Chakrabortee et al. 2012). According to hypothesis (1), G3LEA proteins work as intracellular filaments (cf. the cytoskeleton) by forming α-helical coiled-coil and higher-order superfilaments during dehydration. Moreover, LEA protein filaments could work together with sugar glasses in a manner analogous to steel-reinforced concrete, where the filaments might increase the tensile strength of the amorphous carbohydrate matrix. This model is alternatively called the steel-reinforced concrete model (Goyal et al. 2003). The molecular shield hypothesis (2) proposes that the desiccation-induced aggregation of some proteins can be suppressed in the presence of a molar excess amount of a G3LEA protein (Goyal et al. 2005; Chakrabortee et al. 2012). One interpretation of this observation is that G3LEA proteins reduce the opportunities for collision between potentially aggregating proteins, simply by physical or electrostatic interference (i.e., electrosteric shielding).

Dehydration of the cell should cause an increase in the concentration of intracellular ions. This has potentially damaging effects on cellular components such as proteins and membranes, and it has been proposed that LEA proteins might act to sequester ions. Dure (1993) suggested that polypeptide segments composed of tandem repeats of 11-mer motifs may form dimeric amphiphilic α-helices between which certain ionic species are sandwiched. Subsequently, several studies have demonstrated that salt tolerance in plants is improved by overexpressing G3LEA proteins (e.g., Liu and Zheng 2005; Liu et al. 2010).

15.4.3 The Structure and Function of G3LEA Model Peptides

15.4.3.1 Development of G3LEA Model Peptides

As described above, considerable information has accumulated on the structural and functional properties of native G3LEA proteins. However, it remains unclear how the repeat regions of the 11-mer motif, which comprise the major part of the primary structure, relate to the biological functions of G3LEA proteins. Recently, to elucidate the role of these regions, 22-mer or 44-mer peptides were chemically synthesized with two or four tandem repeats of the consensus 11-mer motifs of G3LEA proteins from several anhydrobiotic organisms (Shimizu et al. 2010). Of these, the most extensively studied peptide is called PvLEA-22, which is a 22-mer model peptide derived from the consensus 11-mer motif of G3LEA proteins in *P. vanderplanki*. Its amino acid sequence is shown in Fig. 15.4c. A control peptide, which we term the "scrambled" peptide (Fig. 15.4c), has an identical amino acid composition but randomized sequence and serves as a comparison. In the subsequent sections, the properties of these peptides will be described in relation to the putative functions of G3LEA proteins.

15.4.3.2 Structure and Thermodynamic Properties of G3LEA Model Peptides

The amide I vibrations of PvLEA-22 and the scrambled peptide both exhibit a spectral pattern characteristic of a disordered structure in D_2O solution. However, on dehydration PvLEA-22 undergoes conformational changes and adopts an α-helical coiled-coil-like structure, whereas the structure of the scrambled peptide remains unchanged (Shimizu et al. 2010). According to recent replica-exchange MD simulations, PvLEA-22 is disordered in water, but on dehydration, two chains of the peptide dimerize in a left-handed coiled-coil arrangement via interactions between the hydrophilic sides of their amphiphilic α-helices (Nishimoto et al. 2017).

Therefore, PvLEA-22 is an intrinsically disordered peptide that behaves similarly to native G3LEA proteins on drying.

PvLEA-22 and the scrambled peptide both vitrify at ambient temperature in the dry state: PvLEA-22 has a T_g of 102 °C, which is higher than that of the scrambled peptide at 84 °C (Shimizu et al. 2010). Furthermore, in the glassy state, PvLEA-22 has a larger activation energy for molecular rearrangements than the scrambled peptide, which means the glassy matrix of the former is kinetically more stable than that of the latter.

PvLEA-22 can form an α-helical coiled-coil structure even in the presence of trehalose, as confirmed for a range of trehalose/PvLEA-22 molar ratios up to 5 (Shimizu et al. 2010). Furthermore, the temperature dependence of the OH and NH stretch vibrations indicates that the T_g values of trehalose/PvLEA-22 mixed samples increase with the relative content of PvLEA-22 (Shimizu et al. 2010). This suggests that the hydrogen bond network in the glassy matrix is reinforced by increasing peptide content. Therefore, the cytoskeleton hypothesis and steel-reinforced concrete model proposed for G3LEA proteins are also valid for the short model peptide studied.

15.4.3.3 Anti-aggregation Effect on Proteins

The effect of PvLEA-22 on desiccation-induced protein aggregation has been tested for several target proteins (Furuki et al. 2012). The turbidity of an aqueous solution of lysozyme significantly increases after desiccation and rehydration, which indicates a considerable amount of aggregation. However, the aggregation is suppressed by PvLEA-22 at lysozyme/peptide molar ratios of 1:2 or higher. The scrambled peptide exhibits almost the same level of protective activity.

More complicated results have been obtained for α-casein (Furuki et al. 2012). A considerable degree of aggregation is caused by the peptide itself when PvLEA-22 is added at lower molar ratios of ~10 relative to α-casein. However, a larger amount of PvLEA-22, added at molar ratios of 25 or 50, suppresses α-casein aggregation. The results with the low molar ratio of PvLEA-22/α-casein can be interpreted as an effect of solution pH change. The addition of PvLEA-22 causes a pH shift of the aqueous solution to ca. 4, close to the isoelectric point, pI (4.2), of α-casein, and consequently the solubility of α-casein is minimized. With the high molar ratios, the poor solubility of α-casein at low pH is thought to be overcome by a shielding effect originating from the addition of excess peptide. Mechanisms of peptide function will be discussed in Sect. 15.4.3.6.

Unexpectedly, a native G3LEA protein (AavLEA1) from a nematode promotes aggregation of lysozyme during desiccation rather than suppresses it (Furuki et al. 2012). Probably, this is due to a strong electrostatic attraction between AavLEA (pI = 5.7) and lysozyme (pI = 11.1). This undesirable result is not observed for a mixture of AavLEA and hydrogenase 2 maturation protease, which is almost the same size as lysozyme, but whose pI is 4.6 (Yamakawa et al. 2013).

PvLEA-22 is capable of maintaining the catalytic activities of enzymes as well as suppressing their aggregation in the dry state (Furuki and Sakurai 2016). While lactate dehydrogenase (LDH, pI = 8.3) is almost inactivated when dried alone, its catalytic activity is preserved at ≥70% of native levels in the presence of PvLEA-22 with molar ratios of PvLEA-22/LDH >500. This degree of protection is comparable to that conferred by several native G3LEA proteins, as reported previously for LDH (Goyal et al. 2005; Grelet et al. 2005; Hatanaka et al. 2013; Boswell et al. 2014; Popova et al. 2015). A similar level of protective activity is also observed for an enzyme with acidic pI, β-D-galactosidase (BDG, pI = 4.6): the catalytic activity is preserved at ≈65% when the peptide is added at a PvLEA-22/BDG molar ratio of 1200 (Furuki and Sakurai 2016).

The scrambled peptide again exhibits almost the same level of protection, in both the above aggregation and enzyme activity tests, as PvLEA-22 (Furuki and Sakurai 2016). Therefore, the amino acid composition of the 11-mer motif is likely responsible for its biological function rather than the specific sequence.

15.4.3.4 Anti-fusion Effect on Liposomes

The effect of PvLEA-22 on dried liposomes has been tested for small unilamellar vesicles (diameter 100 nm) composed of 1-palmitoyl 2-oleoyl-sn-glycero-3-phosphatidylcholine (Furuki and Sakurai 2014). Dynamic light scattering measurements indicate that PvLEA-22 and the scrambled peptide are both capable of protecting against fusion caused by desiccation. Indeed, liposomes maintain their prestress size distribution when these peptides are added at a peptide/POPC molar ratio of more than 0.5. In addition, at a peptide/POPC molar ratio of 0.7, 60% of a fluorescent probe loaded inside the liposomes is retained after dehydration-rehydration treatment. This result is comparable to or better than that of the scrambled peptide and several native LEA proteins.

More recently, the anti-fusion effect of PvLEA-22 was also tested for giant vesicles (diameter 6–9 μm), whose size and phospholipid compositions resemble those of living cells (Furuki and Sakurai 2016). As anticipated, for giant vesicles prepared with egg phosphatidylcholine, PvLEA-22 maintains the vesicular structure in a concentration-dependent manner, preserving about 70% of the total number of vesicles before drying when the peptide is added at 10 mM.

15.4.3.5 Ion-Scavenging Function

The behavior of PvLEA-22 and the scrambled peptide under high-salt conditions (at low water activities) has been investigated by addition of several salts such as NaCl, KCl, $MgCl_2$, and $CaCl_2$ (Furuki et al. 2011). According to circular dichroism (CD) and FTIR measurements, both peptides are disordered in aqueous solution, regardless of whether any salt is present. On the other hand, in the dry state, the majority of these molecules adopt an α-helical conformation when they are mixed with either NaCl or KCl. In contrast, on addition of either $MgCl_2$ or $CaCl_2$, no α-helices are detected, and instead β-sheet structure is formed as well as random coils. These results suggest that both PvLEA-22 and the scrambled peptide are able to scavenge ionic species, so that their conformations are changed depending on the ion species added. The parent LEA protein of PvLEA-22, PvLEA, also exhibits similar conformational transitions depending on the salt added.

15.4.3.6 The Mechanism of Desiccation Protection by LEA Peptides

PvLEA-22 has a characteristic amino acid composition: 12 of the 22 residues are charged amino acids (Glu×2, Asp×4, Lys×6) (Fig. 15.4c). Due to its electrostatic near- neutrality, the peptide is expected to interact with targets such as water-soluble proteins neither too strongly nor too weakly, regardless of the net charge of the target. In addition, PvLEA-22 is disordered in aqueous solution, and thus it is likely to be able to change its conformation flexibly for efficient interaction with a given target. From these characteristic features, it is likely that an excess amount of PvLEA-22 surrounds target molecules in the aqueous solution.

According to quartz crystal microbalance experiments by Yamakawa et al. (2013), the dissociation constant of the PvLEA-22-lysozyme system in aqueous solution is 10^{-5} M, which is comparable to those of various enzyme-substrate systems. MD simulations provide atomistic-level information about how the peptide interacts with a protein or lipid bilayer. In aqueous solution, PvLEA-22 molecules bind to the surface of lysozyme through hydrogen bonds, mainly between Arg (lysozyme)-Glu and Asp (PvLEA-22), which allows shielding of the entire surface except for the cleft region (Fig. 15.5a) (Usui et al. 2014). The interaction between PvLEA-22 and a POPC lipid bilayer surface is shown in Fig. 15.5b, where the side chains of Lys residues penetrate into the bilayer surface, and consequently their positively charged side chains, $-(CH_2)_4NH^{3+}$, directly hydrogen bond with nearby phospholipid head groups (Furuki and Sakurai 2016). This MD result fits very well with the FTIR observation that the asymmetric stretch vibration of the P=O group shifts to a lower wavenumber following addition of PvLEA-22 (Furuki and Sakurai 2014). The binding free energy between PvLEA-22 and the POPC lipid bilayer has been

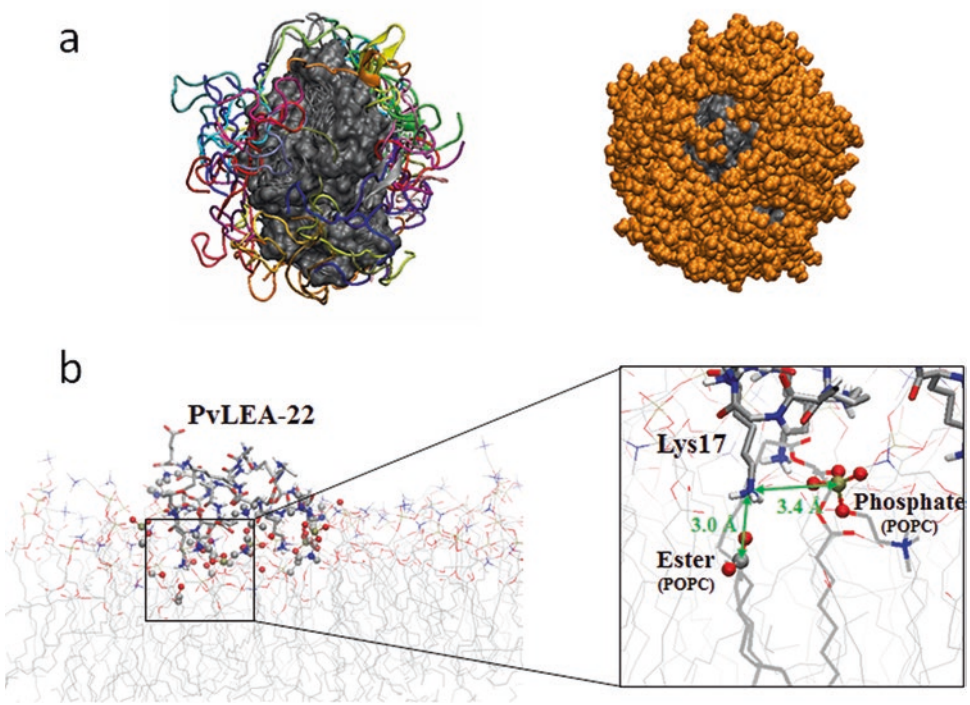

Fig. 15.5 Interaction between PvLEA-22 and a protein or phospholipid bilayer in aqueous solution. (**a**) Lysozome-PvLEA-22 interaction. Left: 45 PvLEA-22 molecules surround one lysozyme molecule (black). Right: the PvLEA-22 molecules are represented by space-filling models. (**b**) The interaction between PvLEA-22 and a POPC lipid bilayer as shown by MD simulation. The inset shows a close-up view, where the NH_3^+ group of Lys17 simultaneously interacts with the phosphate group of a nearby POPC molecule and the ester oxygen atom of a neighboring phospholipid

estimated to be as large as −18 kcal/mol ($K_d = 4.6 \times 10^{-14}$) (Banno et al. 2015), which is comparable to the interaction between an antimicrobial peptide such as melittin and a lipid bilayer (Irudayam and Berkowitz 2012). More importantly, the complex formation shown in Fig. 15.5b causes an increase in the order parameter of the fatty acid chains, contributing to the stabilization of the bilayer (Furuki and Sakurai 2016).

In a dehydrating system, in which water content is gradually decreasing, greater and greater numbers of peptide molecules are expected to bind to the surface of the target and finally to shield it entirely. In this way, peptides might function as a physical barrier that suppresses aggregation during desiccation, as well as substituting bound water, thereby stabilizing the three dimensional structure of the target. According to a simple geometrical consideration, the longitudinal section of one PvLEA-22 molecule (or one scrambled peptide) in an extended conformation is 4.32 nm^2. Using this value, one can estimate the minimum amount of the peptide required to entirely cover the surface of a given enzyme or liposome. For example, the minimum molar ratio of PvLEA-22/LDH required is 100 and that of PvLEA-22/POPC for a 100 nm size vesicle is 0.07. The actual molar ratios of PvLEA-22/target required for protection (see Sects. 15.4.3.5 and 15.4.3.6) are considerably larger than these minimum amounts. Thus, the shielding layer must be more than a single peptide molecule thick; since the protectant is present in excess, it is likely to vitrify as described in Sect. 15.4.3.2, and such a vitrified layer should function as a good entropy barrier.

Usually, IDPs are able to adopt specific conformations required for their biological function

by interacting with a partner protein or ion (Uversky et al. 2000). As described above, PvLEA-22 is a multifunctional peptide, and thus it might adopt different conformations according to each function. Thus, it forms an α-helical coiled-coil on dehydration in the pure state or mixed with trehalose (Shimizu et al. 2010), which is suitable for its putative cytoskeletal function because the coiled-coil structure is rigid and inflexible. In its role as an ion scavenger, the peptide undergoes different structural changes according to the ionic species trapped (Furuki et al. 2011). When shielding the liposome, PvLEA-22 adopts a β-sheet-rich conformation at a low PvLEA-22/POPC molar ratio (= 0.1), as revealed by FTIR measurements (Furuki and Sakurai 2014). This observation is consistent with the MD result of Fig. 15.5b, where the secondary structure analysis indicates a preference for the formation of β-sheet-like fragments (Banno et al. 2015). Currently, it remains unclear what structure the peptides adopt when binding to a protein in the dry state. However, the possibility that the peptides might function in the disordered state cannot be discounted, because the scrambled peptide, which does not exhibit a desiccation-induced increase in defined structure, has almost the same degree of anti-aggregation activity on the proteins studied (Furuki et al. 2012; Furuki and Sakurai 2016).

15.5 Conclusions and Perspectives

Due to the presence of its unique glycosidic bond, the α,α-1,1-linkage, trehalose has a single stable conformation with a clamshell-like shape, which is responsible for its unique bulk properties in both the dry and hydrated states. Its dual functions of a good glass former and a good water substitute are not mutually exclusive and thus render this sugar optimal in its role in anhydrobiosis as an entropy barrier.

The synthetic peptide PvLEA-22 has the ability to preserve proteins/enzymes and liposomes in the dry state, often working as well as native G3LEA proteins. This finding implies that the functional region of native G3LEA proteins is located in the repeated 11-mer motif region. Due to its lack of regular structure and electrostatic neutrality in water, the peptide can interact with various targets. An excess amount of peptide can shield and stabilize the target by acting as a substitute for bound water and as a vitreous physical barrier in the dry state. Therefore, the mechanisms of PvLEA-22 and trehalose function are very similar to each other.

It may be worth mentioning that these molecules exhibit protective activity against environmental stresses other than desiccation. Trehalose also protects against low temperature, high temperature, and oxidation as well as other stresses (Elbein et al. 2003; Oku et al. 2003). Recently, it was found that PvLEA-22 suppresses the thermal denaturation of lysozyme (Furuki and Sakurai in press), although native G3LEA proteins apparently have no ability to prevent heat-induced protein aggregation (Chakrabortee et al. 2012). Honjoh et al. (2008) demonstrated that G3LEA model peptides similar to PvLEA-22 but with >4 11-mer repeating units exhibit good cryoprotective activities.

To date, cryopreservation has been widely used for biomaterials in the biological, medical, and agricultural research fields. However, all cryopreservation techniques require the use of freezers, which in turn require a power supply. Thus, cryopreservation is suboptimal as a long-term solution for the preservation of biomaterials, because of the running cost, potential difficulties involving the safe transfer of materials under storage, and the possibility of fatal cellular damage should an interruption to the electricity supply occur. These problems would be overcome if the target materials could be successfully dried in an active form and stored at ambient temperatures. Trehalose and the G3LEA model peptides can be produced on an industrial scale, which makes it practical to consider their widespread use as protective reagents for the low-cost storage of biomaterials.

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Part III

Application Technologies from Laboratory to Society

Supercooling-Promoting (Anti-ice Nucleation) Substances

16

Seizo Fujikawa, Chikako Kuwabara, Jun Kasuga, and Keita Arakawa

Abstract

Studies on supercooling-promoting substances (SCPSs) are reviewed introducing name of chemicals, experimental conditions and the supercooling capability (SCC) in all, so far recognized, reported SCPSs and results of our original study are presented in order to totally show the functional properties of SCPSs which are known in the present state. Many kinds of substances have been identified as SCPSs that promote supercooling of aqueous solutions in a non-colligative manner by reducing the ice nucleation capability (INC) of ice nucleators (INs). The SCC as revealed by reduction of freezing temperature (°C) by SCPSs differs greatly depending on the INs. While no single SCPS that affects homogeneous ice nucleation to reduce ice nucleation point has been found, many SCPSs have been found to reduce freezing temperatures by heterogeneous ice nucleation with a large fluctuation of SCC depending on the kind of heterogeneous IN. Not only SCPSs increase the degree of SCC (°C), but also some SCPSs have additional SCC to stabilize a supercooling state for a long term to stabilize supercooling against strong mechanical disturbance and to reduce sublimation of ice crystals. The mechanisms underlying the diverse functions of SCPSs remain to be determined in future studies.

Keywords

Supercooling-promoting substance · Anti-ice nucleation substance · Freezing of water · Homogeneous ice nucleation · Heterogeneous ice nucleation · Emulsion freezing · Droplet freezing · Antifreeze proteins and glycoproteins · Polyphenols

Abbreviations

AFGP	Antifreeze glycoprotein protein
AFP	Antifreeze protein
BMQW	Buffered MQ-water
FT_{50}	Temperature at which 50% of the water sample is frozen
IN	Ice nucleator
INB	Ice nucleation bacteria
INC	Ice nucleation capability
MQ-W	Ultrapure water
SCC	Supercooling capability
SCPS	Supercooling-promoting substance

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16.1 Introduction

The phase of water is changed from liquid to solid by a reduction in temperature. Pure water solidifies thermodynamically at 0 °C as an equilibrium freezing point corresponding to the equilibrium melting point. However, initiation of freezing at 0 °C is not kinetically favored in pure water. Pure water is supercooled, in a metastable state, to the temperature limit (supercooling point) of approximately −40 °C, and below that temperature freezes instantly due to homogeneous ice nucleation by clustering of water molecules to form effective ice nucleators (INs) following growth of ice crystals. Achieving freezing of water by homogeneous ice nucleation is usually difficult but is possible to produce experimentally by an emulsion freezing assay using emulsified microdroplets of ultrapure water (MQ-W) (Vali 1995; Fig. 16.1). In most cases, freezing of water occurs at temperatures much higher than −40 °C by heterogeneous ice nucleation due to the presence of diverse kinds of heterogeneous INs in water. Heterogeneous INs enhance freezing at higher subzero temperatures to facilitate clustering of water molecules to form effective INs by their catalyzing action. While many substances have effects as heterogeneous INs, specific heterogeneous INs that have high ice nucleation capability (INC) to facilitate freezing of water at far higher temperatures have been identified (Vali 1995; Fig. 16.1).

In contrast to heterogeneous INs, the existence of anti-INs, which reduce the ice nucleation temperature (freezing point) of water, has recently been reported. Such anti-INs promote supercooling of water at very low concentrations in a non-colligative manner by inhibiting or reducing the effects of INs. Anti-INs that cause a resultant increase of supercooling capability (SCC) in aqueous solutions are also called supercooling-promoting substances (SCPSs). In this review, we will mainly use terminology of SCPSs in opposite to terminology of ice nucleation substances or INs. Studies on SCPSs are important for not only understanding biological mechanisms for successful overwintering but also for technical applications of unfrozen water. However, studies on SCPSs have yet been insufficient. So far recognized, only one review on SCPSs has been published until now (Holt 2003a). Therefore, in addition to reviewing recently published results of SCPSs, our unpublished results regarding new properties of SCPSs are introduced here to provide further information for understanding the characteristics of SCPSs.

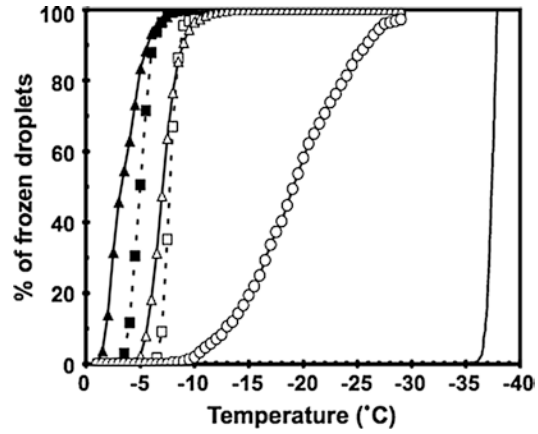

Fig. 16.1 Cumulative freezing spectra in solutions containing silver iodide (black triangle) showing FT_{50} at −4.0 °C, the INB *E. ananas* (black squares) showing FT_{50} at −4.5 °C, INB *X. campestris* (white square) showing FT_{50} at −7.8 °C, phloroglucinol (white triangles) showing FT_{50} at −7.5 °C, and BMQW alone (white circles) showing FT_{50} at −22.0 °C obtained by droplet freezing assays using 2 μL droplets at a cooling rate of 0.2 °C/min observed by the naked eye. A cumulative freezing spectrum in emulsified microdroplet of BMQW alone (line) showing FT_{50} at −37.4 °C obtained by an emulsion freezing assay observed by microscopy. Each spectrum was produced by more than 200 droplets. (From Kuwabara et al. (2012) with permission by ELSEVIER)

16.2 Measurement of SCC

Experimentally, freezing temperatures of water are generally measured by an emulsion freezing or droplet freezing assay (Vali 1995). In the emulsion freezing assay, droplets of emulsified ultrapure water (MQ-W) with diameter of 10 μm

or less (around 0.5 pL) are usually used to determine the homogeneous ice nucleation temperature by preventing the effect of heterogeneous ice nucleation due to unintentionally contained airborne impurities or the effect of surfaces of the container. In the droplet freezing assay, small water droplets of about 1–5 mm in diameter (around 1–10 μL) on a plate are usually used. Freezing of these water droplets by cooling is detected visually or by thermal analysis. Visual observations are done by the naked eye for the droplet freezing assay and by microscopy for the emulsion freezing assay. The number of frozen water droplets is counted as a function of temperature reduction from which a cumulative freezing spectrum is produced. For example, Fig. 16.1 shows cumulative freezing spectra of MQ-W alone obtained by an emulsion freezing assay with freezing spectra corresponding to homogeneous ice nucleation and cumulative freezing spectra of MQ-W containing different heterogeneous INs obtained by a droplet freezing assay showing individual freezing spectra of solutions containing different heterogeneous INs. Even in the droplet freezing assay using small droplets of MQ-W, freezing occurs at temperatures much higher than −40 °C in emulsified droplets of MQ-W by emulsion freezing, indicating the presence of impurities as heterogeneous INs. The freezing temperature (supercooling point) in each sample solution has generally been indicated by the temperature at which 50% of the water sample is frozen (FT_{50}) from the cumulating freezing spectrum. In thermal analysis, a maximum exothermal peak produced by freezing of the sample solution is taken as the freezing temperature (Kuwabara et al. 2012).

The magnitude of SCC (°C) in SCPSs is obtained by the difference between FT_{50} of a control sample (Fig. 16.1 shows several control samples with different INs.) and that of an experimental sample containing diluted SCPSs. In many reports, the temperature difference between control and experimental samples is shown without calibration of osmolality in control samples against experimental samples, in which osmolality is increased by addition of SCPSs. Because of the very low concentration of SCPSs added to experimental samples, however, solute-induced equilibrium freezing (melting) point depression by 1.86 °C/Osm with an accompanying increase of supercooling by one to three times the degree of equilibrium melting point depression (Wilson et al. 2003) in experimental samples has been neglected in many studies. In some studies, although Osm of control solutions was calibrated by adding osmoticum, the effect of calibration was negligible (Kasuga et al. 2007).

On the other hand, it should be noted that in most studies shown in Table 16.1, the degree of SCC (°C) is shown without a significant difference. In such studies, SCC (°C) might have been obtained only from a single set of cumulative freezing spectra in control and experimental samples, though each spectrum was obtained by measurement of more than 100 of water droplets. Studies in which significant differences (mean ± SD) were calculated showed a large fluctuation of SCC among separate experiments (Kuwabara et al. 2012, 2013, 2014; Tables 16.2, 16.3, 16.4, and 16.5). Readers should note that most of the studies on SCPSs are introduced in this review even if statistical significance of SCC is not shown. On the other hand, in addition to studies showing statistical significance of SCC in SCPSs (Tables 16.2, 16.3, 16.4, and 16.5), mean SCC has also been obtained by repeating freeze-warming of the same sample using an automatic lag time apparatus (Wilson and Leader 1995; Table 16.1). In a later study, the cooling rate for each freezing by automatic lag time apparatus was shown to be 1.08 °C/min (Wilson et al. 2003).

16.3 Diversity of Supercooling-Promoting (Anti-ice Nucleation) Substances (SCPSs)

In studies carried out from the early 1980s, many kinds of substances have been identified as SCPSs. Various identified SCPSs of biological origin and synthesized chemicals show degrees of SCC from the range of 0.1 to 15.8 °C

Table 16.1 List of supercooling-promoting substances (SCPSs)

Supercooling-promoting substances	Experimental condition					Supercooling capability (°C)	References
	Concentration of SCPS	INs[a]	Method	Volume of droplets (μL)	Cooling rate (°C/min)		
Biological origin							
Antifreeze glycoproteins							
AFGP from Antarctic cod *Dissostichus mawsoni*	1 mg/mL	INB *Erwinia herbicola*	Droplet freezing	1	Not shown	1.0	Parody-Morreale et al. (1988)
	10 mg/mL	None (airborne ice nucleators in saline solution)	Automatic lag time apparatus using glass tube	3	Not shown (1.08)	1.2	Wilson and Leader (1995)
	10 mg/mL	Protein ice nucleators from hemolymph of New Zealand weta *Hemideina maori*	Automatic lag time apparatus using glass tube	3	Not shown	2.5	
	1% (w/w)	None (airborne ice nucleators in filtrated tap water)	Sample tube	100	0.6	4.9	Holt (2003b)
Antifreeze proteins							
AFP from larvae of the beetle *Dendroides canadensis*	Not shown	Protein ice nucleators from hemolymph of *D. canadensis*	Droplet freezing	1	1.0	1.7	Duman (2002)
	Not shown	INB *Pseudomonas syringae*	Droplet freezing	1	1.0	1.2	
AFP III from ocean pout *Macrozoarces americanus*	0.5% (w/w)	None (airborne ice nucleators in filtrated tap water)	Sample tube	100	0.6	1.5	Holt (2003b)
	0.1% (w/w)	INB *P. syringae* (Snomax) in filtrated tap water	Sample tube	100	0.6	0.3	
	0.01–1.0 mg/mL	Silver iodide	Emulsion freezing	10^{-9}–10^{-5}	0.42	4.5–10.9	Inada et al. (2012)
AFP I from winter flounder *Pleuronectes americanus*	0.01–1.0 mg/mL	Silver iodide	Emulsion freezing	10^{-9}–10^{-5}	0.42	3.8–12.0	
	3–10 mg/mL	None (airborne ice nucleator sin 0.3 M sodium chloride) KUIN-3	Automatic lag time apparatus using glass tube	200	Not shown (1.08)	3.0–5.0	Wilson et al. (2010)

Other protein							
55-kDa protein from Acinetobacter calcoaceticus KINI-1	12.5 μg/mL	INB *Erwinia uredovora* KUIN-3	Droplet freezing	10	1.0	2.2	Kawahara et al. (1996)
Polysaccharide							
130-kDa polysaccharide from Bacillus thuringiensis YY529	50 μg/mL	INB *Pantoea ananas* KUIN-3	Droplet freezing	10	1.0	2.3	Yamashita et al. (2002)
	50 μg/mL	INB *Xanthomonas translucens* IFO 13558	Droplet freezing	10	1.0	1.0	
	50 μg/mL	INB *Pseudomonas fluorescens* KUIN-1	Droplet freezing	10	1.0	0.3	
	50 μg/mL	Silver iodide	Droplet freezing	10	1.0	4.2	
	50 μg/mL	Metaldehyde	Droplet freezing	10	1.0	0.1	
	50 μg/mL	Fluoren-9-one	Droplet freezing	10	1.0	1.1	
	50 μg/mL	Phenazine	Droplet freezing	10	1.0	1.0	
	50 μg/mL	None (airborne ice nucleators in distilled water)	Droplet freezing	10	1.0	0	
Phenylpropanoid							
Eugenol	1 mg/mL	INB *E. uredovora* KUIN-3	Droplet freezing	10	1.0	1.9	Kawahara and Obata (1996)
α-Methoxyphenol	1 mg/mL	INB *E. uredovora* KUIN-3	Droplet freezing	10	1.0	0.2	
2-Allylphenol	1 mg/mL	INB *E. uredovora* KUIN-3	Droplet freezing	10	1.0	2.5	
4-Allylanisole	1 mg/mL	INB *E. uredovora* KUIN-3	Droplet freezing	10	1.0	1.2	
Terpenoid							
Hinokitiol	10 mM	INB *P. fluorescens* KUIN-1	Droplet freezing	10	1.0	2.1	Kawahara et al. (2000)
Hinokitin	10 mM	INB *P. fluorescens* KUIN-1	Droplet freezing	10	1.0	1.1	
α-Pinene	10 mM	INB *P. fluorescens* KUIN-1	Droplet freezing	10	1.0	1.1	
α-Terpinene	10 mM	INB *P. fluorescens* KUIN-1	Droplet freezing	10	1.0	0.4	
Limonene	10 mM	INB *P. fluorescens* KUIN-1	Droplet freezing	10	1.0	1.6	
Flavonol glycoside							
Kaempferol-3-*O*-ß-glucoside	1 mg/mL	INB *Erwinia ananas*	Droplet freezing	2		4.0	Kasuga et al. (2008)
Kaempferol-7-*O*-ß-glucoside	1 mg/mL	INB *E. ananas*	Droplet freezing	2	0.2	9.0	
Quercetin-3-*O*-ß-glucoside	1 mg/mL	INB *E. ananas*	Droplet freezing	2	0.2	2.8	
8-methoxykaempferol-3-*O*-ß-glucoside	1 mg/mL	INB *E. ananas*	Droplet freezing	2	0.2	3.4	

(continued)

Table 16.1 (continued)

Supercooling-promoting substances	Experimental condition					Supercooling capability (°C)	References
	Concentration of SCPS	INs[a]	Method	Volume of droplets (μL)	Cooling rate (°C/min)		
Hydrolyzable tannin							
1,2,6-tri-*O*-galloyl-ß-D-glucopyranose	1 mg/mL	INB *E. ananas*	Droplet freezing	2	0.2	7.3	Wang et al. (2012)
1,2,3,6-tetra-*O*-galloyl-ß-D-glucopyranose	1 mg/mL	INB *E. ananas*	Droplet freezing	2	0.2	8.7	
1,2,3,4,6-penta-*O*-galloyl-α and ß-D-glucopyranose	1 mg/mL	INB *E. ananas*	Droplet freezing	2	0.2	8.0	
2,2′,5-tri-*O*-galloyl-α, ß-D-hamamelose	1 mg/mL	INB *E. ananas*	Droplet freezing	2	0.2	8.5	
Other polyphenols							
See Tables 16.2 and 16.3							
Synthesized chemicals							
Methyl methacrylate and n-Vinylpyrrolidone (MMANVP)	0.0069 g/mL	None (airborne ice nucleators in distilled water)	Droplet freezing	Not shown	Not shown	1.2	Caple et al. (1983a)
	0.0100 g/mL	Silver iodide	Droplet freezing	Not shown	Not shown	2.7	
Poly(vinyl alcohol) (PVA)	0.1–1% (w/w)	INB *P. syringae* (Snomax) in filtrated tap water	Sample tube	100	0.6	0.8–1.4	Holt (2003b)
	0.1–1% (w/w)	None (airborne ice nucleators in filtrated tap water)	Sample tube	100	0.6	2.3–4.0	
	0.1% (w/w)	INB *P. syringae*	Droplet freezing	1	2.0	0.8	Wowk and Fahy (2002)
	0.01–1.0 mg/mL	Silver iodide	Emulsion freezing	10^{-9}–10^{-5}	0.42	10.5–15.8	Inada et al. (2012)

Polyglycerol (PGL)	0.001–1% (w/w)	INB *P. syringae*	Droplet freezing	1	2.0	1.2–6.6	Wowk and Fahy (2002)
Poly(vinylpyrrolidone) (PVP)	0.01–1.0 mg/mL	Silver iodide	Emulsion freezing	10^{-9}–10^{-5}	0.42	5.8–12.8	Inada et al. (2012)
Poly(ethylene glycol) (PEG)	0.01–1.0 mg/mL	Silver iodide	Emulsion freezing	10^{-9}–10^{-5}	0.42	9.1–10.8	
Surfactants							
See, Table 16.4							

See also Tables 16.2, 16.3, and 16.4 for lists of other SCPSs

[a]Except for indicated, INs are dispersed in MQ-water, distilled water, or diluted buffer solution

Table 16.2 List of supercooling-promoting flavonoid compounds showing SCC or INC in solutions containing different INs obtained by droplet freezing[a, b]

Substances	Abbrev.	Conc.(mg/mL)	SCC (°C) or INC (−°C)[c, d]				
			INs				
			E. ananas 2 mg/mL)	*X. campestris* (2 mg/mL)	Silver iodide (0.5 mM)	Phloroglucinol (120 mM)	None (airborne ice nucleators)
Flavonol							
Kaempferol 3-*O*-ß-D-glucopyranoside	K3Glc	0.5	4.7[e]	1.0[e]	4.6[e]	1.0[e]	−2.7[e]
Kaempferol 7-*O*-ß-D-glucopyranoside	K7Glc	0.5	7.2[e]	3.0[e]	2.0[e]	0.0[e]	−12.1[e]
Kaempferol 3-*O*-β-D-rutinoside	K3Rut	1.0	1.4 ± 0.7*	0.1 ± 0.2	4.3 ± 0.7*	3.0 ± 0.4*	−5.3 ± 2.5
Kaempferol 3-*O*-robinoside-7-*O*-rhamnoside	K3Rob7Rha	1.0	3.7 ± 0.5*	0.0 ± 0.2	2.4 ± 0.3*	2.2 ± 1.1	−6.7 ± 0.1
Quercetin 3-*O*-β-D-glucopyranoside	Q3Glc	0.5	3.2[e]	0.9[e]	4.2[e]	4.5[e]	0.8[e]
Quercetin 3-*O*-β-D-galactopyranoside	Q3Gal	1.0	3.3 ± 0.4*	0.6 ± 0.8	4.8 ± 0.8*	2.1 ± 0.6	3.2 ± 2.0
Quercetin 3-*O*-β-D-rhamnopyranoside	Q3Rha	0.5	0.5 ± 0.3	–[f]	3.0 ± 1.3*	2.8 ± 1.9*	–[f]
Quercetin 3-*O*-β-D-rutinoside	Q3Rut	0.5	−0.2 ± 0.2	0.2 ± 0.3	2.0 ± 1.5*	1.7 ± 0.7	−1.6 ± 4.2
Quercetin 5-*O*-β-D-glucopyranoside	Q5Glc	1.0	0.5 ± 0.3	−0.2 ± 0.7	2.9 ± 1.0*	3.5 ± 1.7*	−7.5 ± 1.4*
Quercetin 7-*O*-β-D-glucopyranoside	Q7Glc	1.0	4.2 ± 0.7*	1.6 ± 0.3*	3.2 ± 0.6*	0.6 ± 1.3	−0.6 ± 0.7
Quercetin 7-*O*-β-D-galactopyranoside	Q7Gal	1.0	2.1 ± 0.3*	0.9 ± 0.3*	3.6 ± 0.4*	0.8 ± 1.1	−5.2 ± 1.7*
Quercetin 3′-*O*-β-D-glucopyranoside	Q3′Glc	1.0	1.5 ± 1.0*	0.3 ± 0.2	5.1 ± 0.4*	1.4 ± 1.3	−3.6 ± 1.1
Quercetin 4′-*O*-β-D-glucopyranoside	Q4′Glc	1.0	1.6 ± 1.2*	0.3 ± 0.2	1.6 ± 0.3	0.8 ± 1.0	−2.9 ± 0.9
Quercetin 3,4′-di-*O*-glucopyranoside	Q34′Glc	1.0	−0.1 ± 0.2	−0.1 ± 0.4	4.4 ± 0.8*	1.3 ± 0.7	−6.9 ± 1.7*
Quercetagetin 7-*O*-β-D-glucopyranoside	Qt7Glc	1.0	0.3 ± 0.8	–[f]	3.8 ± 0.8*	–[f]	−5.1 ± 2.3*
Gossypetin 8-*O*-glucopyranoside	G8Glc	1.0	0.1 ± 0.3	2.3 ± 0.8*	3.7 ± 1.1*	−0.1 ± 0.4	−2.6 ± 1.3
Myricetin 3-*O*-β-D-glucopyranoside	M3Glc	1.0	1.6 ± 1.0	0.1 ± 0.1	3.4 ± 0.6*	−0.8 ± 0.5	2.7 ± 3.1
Myricetin 3-*O*-α-L-rhamnopyranoside	M3Rha	1.0	0.1 ± 0.3	0.1 ± 0.1	4.0 ± 0.4*	0.9 ± 0.9	−4.6 ± 2.3
Syringetin 3-*O*-galactopyranoside	S3Gal	1.0	0.3 ± 0.5	−0.2 ± 0.4	5.8 ± 0.7*	0.2 ± 0.6	−2.1 ± 1.7

Morin 7-*O*-β-D-galactopyranoside Et3N salt (1:1)	M7Gal	0.5	0.1 ± 0.1	0.6 ± 0.7	4.1 ± 0.8*	−0.9 ± 0.7	−5.2 ± 5.2
α-oligoglucosyl quercetin 3-*O*-β-D-glucopyranoside	Q3(Glc)n	1.0	4.1 ± 1.4*	0.2 ± 0.6	5.8 ± 0.6*	2.4 ± 0.5*	2.8 ± 2.3
4^G-α-D-glucopyranosyl rutin	αG-Rut	1.0	0.3 ± 0.2	0.2 ± 0.2	5.9 ± 0.3*	1.6 ± 0.4	2.4 ± 1.7
Quercetin 3-*O*-(6″-acetyl)-β-D-galactopyranoside	Q3–6″Gal	1.0	0.3 ± 0.5	0.2 ± 0.2	5.5 ± 1.0*	3.2 ± 2.8*	−0.1 ± 2.2
Quercetin 5-(3,6,9-trioxadecanyl) ether	Q5Tri	1.0	−0.2 ± 0.1	0.0 ± 0.3	4.2 ± 0.4*	−0.6 ± 1.3	−3.5 ± 2.4
Flavone							
Apigenin 7-*O*-β-D-glucopyranoside	A7Glc	1.0	2.1 ± 0.3*	0.2 ± 0.4	0.4 ± 0.1	0.5 ± 1.0	−4.7 ± 2.3
Flavanone							
4^G-α-D-glucopyranosyl hesperidin	G-Hes	1.0	0.2 ± 0.3	–[f]	5.0 ± 0.7*	–[f]	−8.8 ± 2.6*
Anthocyanidin							
Pelargonidin 3-*O*-glucopyranoside chloride	P3Glc	1.0	1.3[e]	0.6 ± 0.3	7.1 ± 1.5*	2.4 ± 1.1*	−6.5 ± 2.1*

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[a]INs are dispersed in MQ-water or distilled buffer solution

[b]Droplet freezing is done using 2 μL droplets cooled at 0.2 °C/min

[c]Presence of significant difference of SCC or INC by difference of FT50 between control and experimental samples was determined by a Dunnett's test and indicated by asterisks (*$p < 0.05$)

[d]Each value is the mean ± SD from 5 separate droplet freezing assay with more than 20 droplets in each assay

[e]Data from a cumulative spectrum in a total of 200 droplets

[f]Not examined

Table 16.3 List of supercooling-promoting tannin-related compounds showing SCC or INC in solutions containing different INs obtained by droplet freezing[a, b]

Substances	Abbrev.	Conc. (mg/mL)	SCC (°C) or INC (−°C)[c, d] INs				
			E. ananas (2 mg/mL)	*X. campestris* (2 mg/mL)	Silver iodide (0.5 mM)	Phloroglucinol (120 mM)	None (airborne ice nucleators)
Hydrolyzable tannins							
1,2,6-tri-*O*-galloyl-ß-D-glucopyranose	tri-GGlc	1.0	1.5 ± 0.4*	0.6 ± 0.5	3.3 ± 0.9*	0.2 ± 0.3	5.1 ± 3.0*
1,2,3,6-tetra-*O*-galloyl-ß-D-glucopyranose	tet-GGlc	1.0	2.9 ± 0.5*	3.0 ± 0.6*	3.7 ± 0.8*	1.0 ± 0.7	4.3 ± 2.5
1,2,3,4,6-penta-*O*-galloyl-α and ß-D-glucopyranose	pent-GGlc	1.0	3.0 ± 0.2*	–[e]	2.2 ± 1.5	6.8 ± 3.7*	1.3 ± 2.8
1-methyl-2,3,4,6-tetra-*O*-galloyl-α-D-glucopyranose	M-tet-GGlc	1.0	3.4 ± 0.2*	–[e]	2.6 ± 1.2*	5.1 ± 2.5*	1.1 ± 1.3
2,2′,5-tri-*O*-galloyl-α,ß-D-hamamelose	tri-GHam	1.0	2.3 ± 0.7*	1.2 ± 0.3*	4.5 ± 0.6*	0.2 ± 0.4	4.3 ± 2.8
Myo-inositol pergallate	MIPG	1.0	3.0 ± 0.2*	–[e]	3.0 ± 0.9*	0.6 ± 1.1	−0.4 ± 4.0
Catechin derivatives							
(+)-catechin	C	1.0	0.9 ± 0.4*	0.3 ± 0.4	4.2 ± 0.2*	0.4 ± 0.7	−9.0 ± 0.8*
(−)-epicatechin	EC	1.0	0.7 ± 0.3	0.2 ± 0.4	4.7 ± 0.7*	0.2 ± 0.6	−9.8 ± 1.0*
(−)-epigallocatechin	EgaC	1.0	0.8 ± 0.4*	−0.1 ± 0.5	4.5 ± 0.3*	1.0 ± 0.4	−7.6 ± 1.1*
(−)-epicatechin gallate	ECG	1.0	3.3 ± 0.3*	0.9 ± 0.3*	4.0 ± 1.7*	1.7 ± 0.5	2.3 ± 2.0
(−)-epigallocatechin gallate	EgaCG	1.0	3.3 ± 0.3*	0.9 ± 0.4*	3.6 ± 1.6*	0.5 ± 0.8	2.3 ± 2.6
(−)-gallocatechin gallate	gaCG	1.0	3.2 ± 0.2*	2.3 ± 0.5*	3.3 ± 1.3*	−0.2 ± 0.4	2.3 ± 2.3
(+)-catechin gallate	CG	1.0	0.4 ± 0.1	–[e]	5.8 ± 0.9*	–[e]	0.7 ± 5.8
Procyanidin A2	PCA2	1.0	0.8 ± 0.1*	–[e]	4.1 ± 2.1*	–[e]	1.5 ± 1.3
Procyanidin B1	PCB1	1.0	1.2 ± 0.2*	–[e]	2.5 ± 0.8*	–[e]	−4.6 ± 3.8
Theaflavin	TF	1.0	1.9 ± 0.3*	–[e]	4.5 ± 0.7*	–[e]	−11.0 ± 2.1*
Theaflavin-3,3′-digallate	TF-diG	1.0	2.6 ± 0.2*	–[e]	2.9 ± 1.1*	–[e]	−5.7 ± 4.1
Structural analogs of catechin							
Ampelopsin 3-gallate	Amp3G	1.0	0.1 ± 0.3	–[e]	4.6 ± 0.6*	–[e]	−1.4 ± 5.9
Phenolcarboxylic acid derivatives							
Methyl gallate	MG	1.0	−0.5 ± 0.4	–[e]	3.3 ± 1.2*	–[e]	−2.7 ± 1.7
Rosmarinic acid	RA	1.0	0.7 ± 0.2	–[e]	3.0 ± 0.3*	–[e]	−10.7 ± 2.0*

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[a]INs are dispersed in MQ-water or distilled buffer solution

[b]Droplet freezing is done using 2 μL droplets cooled at 0.2°C/min

[c]Presence of significant difference of SCC or INC by difference of FT50 between control and experimental samples was determined by a Dunnett's test and indicated by asterisks (*$p < 0.05$)

[d]Each value is the mean ± SD from 5 separate droplet freezing assay with more than 20 droplets in each assay

[e]Not examined

Table 16.4 List of supercooling-promoting surfactants showing SCC or INC in solutions containing different INs obtained by droplet freezing[a, b]

Substances	Abbrev.	Conc. (mg/mL)	Molarity[c]	SCC (°C) or INC (−°C)[d, e]		
				INs		
				E. ananas (2 mg/mL)	Silver iodide (0.5 mM)	None (airborne ice nucleators)
Nonionic surfactants						
Triton X-100	TX-100	0.1	–[f]	0.1 ± 0.3	6.9 ± 0.6*	−0.9 ± 1.4
Triton X-114	TX-114	0.1	–[f]	−0.4 ± 0.4	7.1 ± 0.6*	−0.2 ± 0.7
Nonidet P-40	NP40	0.1	–[f]	0.0 ± 0.2	10.9 ± 2.4*	−1.5 ± 2.5
Tween 20	TW20	0.1	–[f]	−0.4 ± 0.6	6.7 ± 1.0*	−0.4 ± 1.0
Tween 80	TW80	0.1	–[f]	0.1 ± 0.2	5.0 ± 1.3*	0.4 ± 0.6
Polyethylene glycol monostearate	PEGMS40	0.1	–[f]	0.1 ± 0.2	9.3 ± 1.7*	−1.2 ± 1.1
Emulgen 2025G	E2025G	0.1	–[f]	0.1 ± 0.3	11.5 ± 2.3*	−0.4 ± 1.3
Rheodol TW-P 120	TWP120	0.1	–[f]	−0.1 ± 0.2	6.6 ± 2.5*	−0.6 ± 1.7
Rheodol SP-L 10	SPL-10	0.1	–[f]	−0.1 ± 0.1	4.5 ± 0.5*	0.4 ± 0.7
Aminon PK 02S	PK02S	0.1	–[f]	0.1 ± 0.1	7.8 ± 2.7*	−0.6 ± 0.2
Megaface F-477	F477	0.1	–[f]	0.1 ± 0.2	9.0 ± 1.8*	−2.2 ± 2.2
Anionic surfactants						
Sodium cholate	SC	0.1	0.2	0.1 ± 0.2	4.3 ± 1.7*	2.2 ± 2.2
Dodecene-1 LAS	D1LAS	0.1	0.3	0.6 ± 0.3*	3.9 ± 1.1*	−0.9 ± 1.2
Cationic surfactants						
Hexyltrimethylammonium bromide	C6TAB	0.1	0.4	0.0 ± 0.1	6.3 ± 0.6*	−0.1 ± 1.7
Hexadecyltrimethylammonium bromide	C16TAB	0.1	0.3	0.6 ± 0.2*	11.8 ± 1.9*	0.3 ± 1.6
Hexadecyltrimethylammonium chloride	C16TAC	0.1	0.3	0.5 ± 0.3*	11.8 ± 0.8*	0.3 ± 1.1
Amphoteric surfactants						
Amphitol 20 N	20 N	0.1	–[f]	0.0 ± 0.2	7.3 ± 0.7*	−0.3 ± 2.1

(continued)

Table 16.4 (continued)

Substances	Abbrev.	Conc. (mg/mL)	Molarity[c]	SCC (°C) or INC (−°C)[d, e]		
				INs		
				E. ananas (2 mg/mL)	Silver iodide (0.5 mM)	None (airborne ice nucleators)
Amphitol 20AB	20AB	0.1	–[f]	−0.1 ± 0.1	4.1 ± 0.7*	0.6 ± 1.8
Myristyl sulfobetaine	MS	0.1	0.3	−0.1 ± 0.2	10.3 ± 0.5*	−1.3 ± 1.5
CHAPS	CHAPS	0.1	0.2	0.0 ± 0.1	7.1 ± 2.3*	−0.2 ± 2.0

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[a]INs are dispersed in MQ-water or distilled buffer solution

[b]Droplet freezing is done using 2 μL droplets cooled at 0.2°C/min

[c]Molarity (mM) was shown only in surfactants that chemical structures were published

[d]Presence of significant difference of SCC or INC by difference of FT50 between control and experimental samples was determined by a Dunnett's test and indicated by asterisks (*$p < 0.05$)

[e]Each value is the mean ± SD from 5 separate droplet freezing assay with more than 20 droplets in each assay

[f]Not examined

Table 16.5 List of supercooling-promoting polyphenol mixtures and crude tannin extracts showing SCC or INC in solutions containing different INs obtained by droplet freezing[a, b]

Substances	Abbrev.	Conc. (mg/mL)	SCC (°C) or INC (−°C)[c, d] INs					Component
			E. ananas (2 mg/mL)	*X. campestris*(2 mg/mL)	Silver iodide(0.5 mM)	Phloroglucinol (120 mM)	None (airborne ice nucleators)	
Polyphenol mixtures								
Tannic acid	TA	1.0	2.4 ± 0.4*	0.5 ± 0.1	4.8 ± 0.8*	1.8 ± 2.0	1.7 ± 0.9	Hydrolyzable gallotannins extracted from Chinese galls for use as a chemical reagent, containing mainly 1,2,3,4,6-pentagalloyl-ß-D-glucopyranose
Tea catechin	TC	1.0	2.6 ± 0.5*	0.7 ± 0.2	8.5 ± 1.4*	1.3 ± 0.3	−5.3 ± 2.7*	Extracts from green tea for use as a dietary supplement, containing more than 40% tea catechin
Cameria extract	Cam	1.0	2.0 ± 0.6*	–[e]	7.1 ± 0.8*	−0.9 ± 1.1	2.2 ± 1.7	Mixture of tea catechins extracted from cameria for use as a dietary supplement, containing 11% EgaCG, 11% EgaC and small amounts of gaC, EC, ECG, gaCG, and C
Sunphenon 90S	S90	1.0	3.3 ± 0.3*	–[e]	4.1 ± 1.1*	0.1 ± 0.8	4.6 ± 2.6*	Mixture of tea catechins extracted from green tea for use as a dietary supplement, containing 50% EgaCG and small amounts of ECG, EgaC, EC, caffeine, gaCG, gaC, and C
Sunphenon 70H-T	S70	1.0	3.2 ± 0.3*	–[e]	5.9 ± 1.5*	–[e]	2.6 ± 2.1	Mixture of tea catechins extracted from green tea for use as a dietary supplement, containing 30% EgaCG and small amounts of EgaC, ECG, EC, gaC, C, and gaCG
Sunphenon XLB-100	SXLB	1.0	1.2 ± 0.4*	–[e]	3.7 ± 0.5*	–[e]	−1.0 ± 4.3	Mixture of tea catechins extracted from green tea for use as a dietary supplement, containing 54% EgaC and small amounts of EC, gaC, C, EgaCG, and gaCG
Sunphenon EGCg	SEgaCG	1.0	3.2 ± 0.4*	–[e]	2.9 ± 1.1*	−0.2 ± 0.8	3.8 ± 1.5*	Mixture of tea catechins extracted from green tea for use as a dietary supplement, containing 95% EgaCG and small amounts of ECG, C, and gaCG

(continued)

Table 16.5 (continued)

Substances	Abbrev.	Conc. (mg/mL)	SCC (°C) or INC (−°C)[c, d] INs					Component
			E. ananas (2 mg/mL)	*X. campestris*(2 mg/mL)	Silver iodide(0.5 mM)	Phloroglucinol (120 mM)	None (airborne ice nucleators)	
Litchee fruit polyphenol	LFP	1.0	1.7 ± 0.6*	1.3 ± 0.6*	5.7 ± 1.6*	0.1 ± 0.3	1.2 ± 1.3	Condensed tannins extracted from dried fruits of lychee by water, containing 20% of low-molecular procyanidin A1, A2 and 80% of other polyphenol compounds such as polymeric A-type procyanidin
Grape seed polyphenol	GSP	1.0	1.2 ± 0.7*	1.1 ± 0.3*	5.5 ± 1.6*	−0.1 ± 0.3	−3.7 ± 4.5	Condensed tannins extracted from seeds of grape by 80% MeOH, containing 10% low-molecular procyanidins and 90% of other polyphenol compounds such as proanthocyanidin polymers
Oligonol	OLG	1.0	2.4 ± 0.2*	1.4 ± 0.9*	4.7 ± 1.0*	−0.1 ± 0.5	2.8 ± 1.3	Mixture of low-molecular-weight processed phenolic products consisting of (_or_ rich in) chemically converted condensed tannins from lychee fruit and tea catechins for use as a dietary supplement, containing approximately 40% catechin type monomer and a small amount of proanthocyanidin oligomers such as EC, procyanidin A2 and EC-EgaCG
Oligonol-X	OLGX	1.0	2.5 ± 0.1*	–[e]	3.3 ± 1.4*	–[e]	3.7 ± 2.0*	Commercially further purified Oligonol for additives to cryoprotectants
Crude plant tannin extracts								
Chestnut tannin	CT	1.0	2.4 ± 0.6*	1.5 ± 0.2*	5.5 ± 0.8*	0.8 ± 0.8	−9.2 ± 0.8*	Crude tannins extracted from wood (bark and timber) of chestnut by hot water for use as a tanning that mainly consist of hydrolyzable tannins, containing castalagin, vescalagin, castalin, and vescalin

Quebracho tannin	QT	1.0	2.3 ± 0.2*	1.4 ± 0.3*	5.7 ± 0.9*	0.8 ± 0.5	−2.9 ± 1.9	Crude tannins extracted from heartwood of quebracho by hot water for use as a tanning that mainly consist of condensed tannins, containing proanthocyanidins as oligomers of C and fisetinidol
Mimosa tannin	MT	1.0	2.6 ± 0.5*	–[e]	3.8 ± 1.0*	–[e]	−3.4 ± 2.8	Crude tannins extracted from bark of mimosa by hot water for use as a tanning that mainly consist of condensed tannins, containing polymeric robinetinidin and profisetinidin
Lannea coromandelica tannin	LT	1.0	2.2 ± 0.3*	–[e]	2.7 ± 0.9*	–[e]	−4.4 ± 3.4	Crude tannins extracted from bark of *Lannea coromandelica* by 80% acetone and 60% MeOH that mainly consist of condensed tannins, containing flavan-3-ols repeating units
Persimmon tannin	PT	1.0	0.9 ± 0.5*	–[e]	4.6 ± 0.6*	–[e]	−6.1 ± 3.0*	Crude tannins extracted from immature fruits of persimmon by water that mainly consist of condensed tannins, containing polymeric proanthocyanidins as oligomers of C, gaC, CG, and gaCG

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[a]INs are dispersed in MQ-water or distilled buffer solution

[b]Droplet freezing is done using 2 μL droplets cooled at 0.2 °C/min

[c]Presence of significant difference of SCC or INC by difference of FT50 between control and experimental samples was determined by a Dunnett's test and indicated by asterisks (*$p < 0.05$)

[d]Each value is the mean ± SD from 5 separate droplet freezing assay with more than 20 droplets in each assay

[e]Not examined

(Tables 16.1, 16.2, 16.3, and 16.4). Other than the SCPSs listed in Tables 16.1, 16.2, 16.3, and 16.4, some studies have shown the presence of SCPSs that were identified by methods different from those described above (Sect. 16.2) or the presence of causable substances that were not identified. As far as we know, although the causative substances were not identified, Layton et al. (1980) has provided the first report on the presence of SCPSs in plant crude extracts. They showed SCC of 2–3 °C in filtrated distilled water extracts from jojoba beans and peach seeds by the droplet freezing assay using 10 μL droplets at a cooling of 1 °C/min. They also showed that distilled water extracts from apricot seeds reduced ice nucleation of silver iodide dispersion by about 3 °C. Caple et al. (1983b) carried out a more extensive study on SCC in seed extracts from peach, apricot, plum, cherry, jojoba, and flax. The resulting distilled water extracts showed SCC of 3–4 °C. Supernatants from extracts of germinated pea seeds exhibited SCC of 8.1 °C. Unfortunately, however, the concentrations of crude plant extracts used for examination were not shown in these reports. In later studies, many SCPSs were identified in plant extracts including phenylpropanoids (Kawahara and Obata 1996), terpenoids (Kawahara et al. 2000), flavonol glycosides (Kasuga et al. 2008), and hydrolysable tannins (Wang et al. 2012) (Table 16.1). The crude plant extracts studied by Layton et al. (1980) and Caple et al. (1983b) might have contained these identified SCPSs. An SCC was also found in crude extracts from xylem of boreal trees (Kasuga et al. 2007), in which their xylem parenchyma cells adapt to winter cold by deep supercooling to around −40 °C (Fujikawa et al. 2009). By droplet freezing assays using 2 μL droplets at a cooling rate of 0.2 °C/min in the presence of ice-nucleating bacteria (INB) *Erwinia ananas*, crude xylem extracts from several boreal trees, including *Betula platyphylla*, *Castanea crenata*, *Fagus crenata*, *Morus bombycis*, *Sorbus commixta*, and katsura-tree *Cercidiphyllum japonicum*, in concentration of 100 mOsm/kg showed SCC of 1.0, 1.2, 1.3, 0.3, 1.0, and 1.9 °C, respectively, in difference of FT_{50} with that of control in which osmolality was calibrated by addition of the same concentration of glucose (Kasuga et al. 2007). From the crude extracts of xylem of the katsura-tree, four flavonol glycosides (Kasuga et al. 2008) and four hydrolysable tannins (Wang et al. 2012) were identified as SCPSs (Table 16.1). As related substances in these supercooling-promoting polyphenols identified from the katsura-tree, many species of flavonoid compounds (Kuwabara et al. 2012; Table 16.2) and tannin-related compounds (Kuwabara et al. 2013; Table 16.3) were identified as SCPS. Polyphenol mixtures and crude tannin extracts from several species of plants also showed SCC (Kuwabara et al. 2013; Table 16.5).

Some antifreeze proteins (AFPs) and antifreeze glycoproteins (AFGPs) have been reported to promote supercooling in the range of 1.0–4.9 °C (Parody-Morreale et al. 1988; Wilson and Leader 1995; Duman 2002; Holt 2003b; Wilson et al. 2010; Inada et al. 2012) (Table 16.1). Olsen and Duman (1997) used a droplet freezing assay and examined the effect of an AFP from the overwintering pyrochroid beetle *Dendroides canadensis* on freezing of a solution containing INB *Pseudomonas fluorescens*. They showed that when this AFP was mixed with the INB *P. fluorescence*, the cumulative ice nucleation spectrum was shifted to a lower temperature, indicating an inhibitory effect of the AFP on ice nucleation resulting in the promotion of supercooling.

Some bacteria-related substances promote supercooling by inhibiting ice nucleation. Kiprianova et al. (1995) examined the ice nucleating capability (INC) of various bacteria in distilled water by droplet freezing and found that some strains of bacteria inhibited ice nucleation. This property was found most frequently in non-fluorescent *Pseudomonas* species. They suggested that the anti-ice nucleation activity was probably due to bacterial metabolites secreted into the medium. The 55 kDa proteins secreted from *Acinetobacter calcoaceticus* (Kawahara et al. 1996) and 130-kDa polysaccharides secreted from *Bacillus thuringiensis* (Yamashita et al. 2002) were shown to have SCC (Table 16.1).

Some synthesized chemicals exhibit SCC. Soon after the original discovery of fish AFPs (Devries and Wohlschlag 1969), Klotz

(1970) speculated that many polymeric molecules including proteins should be able to inhibit freezing by selective binding to heterogeneous INs. Caple's group (1983a) synthesized such a polymer, MMANVP, with anti-nucleating properties by imitation of the hydrophobic-hydrophilic character of AFGP molecules (Table 16.1). Similarly, by focusing on the hydrophobic-hydrophilic character of many reported SCPSs, Kuwabara et al. (2014) found SCC in a variety of surfactants (Table 16.4). Wowk et al. (2000) showed anti-ice nucleation capacity of PVA by using a differential scanning calorimeter, showing that addition of 0.1% (v/w) PVA can reduce the concentrations of glycerol and dimethyl sulfoxide for vitrification. Visual observation also showed that 1% PVA inhibited ice crystal growth in vitrified 58% ethylene glycol (EG) solutions during devitrification upon warming (Wowk et al. 2000). In addition to measurement of SCC by a droplet freezing assay (Table 16.1), Wowk and Fahy (2002) showed that 1% PVA and 1% PGL reduced the number of ice nucleation sites by visually counting the approximate number of ice crystals formed in a 15 g solution of 55% EG containing INB *Pseudomonas syringae* cooled to −130 °C. Inada et al. (2012) showed that PEG and PVP exhibited SCC (Table 16.1). A synthesized polyphenol, OLG, also exhibited SCC (Table 16.5).

16.4 Effect of SCPSs Against Different Kinds of INs

16.4.1 Effect to Homogeneous Ice Nucleation

Although diverse kinds of SCPSs have been identified (Tables 16.1, 16.2, 16.3, and 16.4), SCPSs that affect homogeneous ice nucleation to reduce the temperature have not been discovered. Franks et al. (1987) examined the effect of AFGP from blood serum of the cod icefishes *Notothenia neglecta* and the effect of PVP on homogeneous ice nucleation by emulsion freezing assays using droplets of 2.5 μm in mean radius with a cooling rate of 1.25 °C/min. AFGP and PVP had similar effects. The addition of 1% of each of these substances to distilled water or to a solution containing sucrose, sodium chloride (NaCl), or fructose did not affect the temperature corresponding to the maximum nucleation rate, indicating that these substances had no effect to promote supercooling on homogeneous ice nucleation. It should be noted that while PVP is now known as an SCPS that reduces freezing temperature against a heterogeneous IN (Inada et al. 2012; Table 16.1), it has not been examined whether AFGP from *N. neglecta* are SCPSs against solutions containing heterogeneous INs.

Kasuga et al. (2007) examined the effect of crude xylem extracts from the katsura-tree on homogeneous ice nucleation by emulsion freezing using emulsified microdroplet around 10 μm in diameter at a cooling of 0.2 °C/min. A maximum exothermal peak by freezing of emulsions obtained by differential thermal analysis was −37.0 ± 0.6 °C in a diluted phosphate buffer solution (BMQW), which may correspond to the homogeneous ice nucleation temperature of water, −38.0 ± 0.6 °C in diluted BMQW including 100 mOsm/kg of glucose (Glc), which has no SCC, and −37.8 ± 0.3 °C in diluted BMQW containing 100 mOsml/kg of crude xylem extracts. These results show that crude xylem extracts do not affect homogeneous ice nucleation, although the crude extracts exhibit SCC in solution containing INB *Erwinia ananas* (see, Sect. 16.2). In emulsion freezing using the same experimental conditions as these described above, the lack of ability to reduce homogeneous ice nucleation temperature was also confirmed for K3Glc, K7Glc, and Q3Glc at a concentration of 0.02% (w/v) (Kuwabara et al. 2011), which have SCC against diverse kinds of heterogeneous INs (Tables 16.1 and 16.2). Emulsion freezing assays also showed that all of the flavonoid compounds examined, including Q3Gal, Q3 (Glc) n, αG-Rut, K3Rob7Rha, Q3Rut, K7Gal, and Q34'Glc, at a concentration of 0.1% (w/v) did not reduce homogeneous ice nucleation temperature (Kuwabara et al. 2012), although these compounds exhibited SCC against many heterogeneous INs (Table 16.2). Emulsion freezing assays

using the same conditions as these described above also showed that all of the tannin-related compounds examined, including pent-GGlc, CA, ChlA, and RA, at a concentration of 0.1% (w/v), and all of the polyphenol mixtures and crude tannin extracts examined, including TA, S90, SEgaCG, LFP, OLG, and CT, at a concentration of 0.1% (w/v), did not reduce the homogeneous ice nucleation temperature (Kuwabara et al. 2013), although they exhibited SCC against many heterogeneous INs (Tables 16.3 and 16.5). Emulsion freezing assays also showed the lack of an effect to reduce the homogeneous ice nucleation temperature in surfactants examined, including E2025G and C16TAB, at a concentration of 0.1% (v/v) (unpublished result), which exhibited SCC against heterogeneous INs (Table 16.4). From all of these results, it seems likely that the SCPSs so far discovered do not directly inhibit the clustering of water molecules for homogeneous ice nucleation, at least at very low temperatures of about −40 °C.

16.4.2 Effects to Heterogeneous Ice Nucleation

16.4.2.1 Variation in Responses of SCPSs to Different Kinds of INs

While SCPSs do not promote supercooling in homogeneous ice nucleation, SCPSs can promote supercooling in solutions containing diverse kinds of heterogeneous INs. The responses of SCPSs against different INs are diverse: (1) the magnitude of SCC (°C) changes depending on the kind of INs, (2) many SCPSs that show SCC against some INs have no SCC against other INs, and (3) SCPSs that show SCC against some INs exhibit ice nucleation capability (INC) against other INs. Therefore, the terminology of SCPSs is used when substances show SCC at least to one kind of IN.

130-kDa Polysaccharide

While many SCPSs have been identified by examination of one kind of IN (Table 16.1), a systematic study to determine the effects of a 130-kDa polysaccharide against eight kinds of INs was conducted (Yamashita et al. 2002). Although the magnitude of SCC was low, it was shown that the 130-kDa polysaccharide exhibited various degrees of SCC against seven kinds of identified heterogeneous INs in the range of 0.1–4.2 °C. It was also shown that 130-kDa polysaccharides lacked SCC against MQ-W alone, which may contain unidentified airborne heterogeneous INs (Table 16.1).

AFPs and AFGPs

AFGPs from the Antarctic cod *Dissostichus mawsoni* in studies by Parody-Morreale et al. (1988), Wilson and Leader (1995), and Holt (2003b) and AFP III from ocean pout *Macrozoarces americanus* in studies by Holt (2003b) and Inada et al. (2012) showed different magnitudes (°C) of SCC against different INs, although the experimental conditions used in those studies were different (Table 16.1). A difference in the magnitude of SCC was also shown in AFP from larvae of the beetle *D. Canadensis* against two different INs (Duman 2002; Table 16.1). It was shown by a droplet freezing assay that although substances believed to be AFPs in the Antarctic nematode *Panagrolaimus davidi* inhibited ice nucleation of INs existing in hemolymph of the New Zealand alpine cockroach, these AFPs did not inhibit ice nucleation by silver iodide (Wharton and Worland 1998). AFPs from larvae of the beetle *D. Canadensis* showed SCC against protein INs and INB *P. syringae* (Table 16.1) but did not inhibit the activity of hemolymph lipoprotein INs from larvae of the crane fly *Tipula trivittata* (Wu and Duman 1991). Although many AFPs and AFGPs show SCC (Table 16.1), it is not clear whether all AFPs and AFGPs, which have the ability to bind nascent ice crystals to inhibit growth of ice crystals, have SCC or not. It has been reported, however, that not all AFPs and AFGPs inhibit INC against all INs. Wu and Duman (1991) showed that a purified AFP from larvae of the beetle *D. canadensis* did not inhibit activity of hemolymph lipoprotein INs from larvae of the crane fly *T. trivittata*. Baust and Zachariassen (1983) showed that INC in INs associated with the cellular

matrix in the cerambycid beetle *Rhagium inquisitor* was not affected by addition of a highly active AFP with thermal hysteresis of 5 °C.

Synthesized Chemicals

The synthesized chemical PVA in studies by Wowk and Fahy (2002), Holt (2003b), and Inada et al. (2012) showed different magnitudes of SCC (°C) against different INs including INB *P. syringae*, tap water, and silver iodide, although the experimental conditions used in these studies were different (Table 16.1). Furthermore, the effects of PVA against seven defined organic INs, including phloroglucinol, 3-aminophenol, phenazine, metaldehyde, phthalic anhydride, acetoacetamide, and 2-nitrodiphenylamine, were examined by visually counting the approximate number of ice crystals that developed in a flask containing 10 mL glass forming 55% (w/w) ethylene glycol (EG) solution cooled to −130 °C. It was shown that PVA was effective against five of the seven INs to inhibit or reduce ice crystal formation (Wowk and Fahy 2002). On the other hand, it was shown in the same experiment that synthesized chemical PGL, which promotes SCC against INB *P. syringae,* (Table 16.1), is an ineffective inhibitor for all of the seven organic INs, suggesting specificity of PGL only to INB *P. syringae* (Wowk and Fahy 2002). The synthesized chemical MMANVP also showed different degrees of SCC (°C) against two INs (Caple et al. 1983a; Table 16.1). Many species of surfactants showed statistically significant SCC with high specificity against a solution containing silver iodide, although a few surfactants including D1LAS, C16TAB, and C16TAC also exhibited statistically significant SCC against INB *E ananas*. No surfactants affecting SCC of MQ-W alone exist (Kuwabara et al. 2014; Table 16.4).

Polyphenol Compounds

Identified polyphenol compounds exhibit large fluctuation of SCC depending on the INs (Tables 16.2 and 16.3). The fluctuation of SCC in these supercooling-promoting polyphenols includes not only degrees of SCC (°C) as well as the effectiveness of SCPSs against different INs but also negative effects of SCPSs on INC depending on INs. Among flavonoid compounds examined against three or five INs, including INB *E. ananas*, INB *Xanthomonas campestris*, silver iodide, phlorologlucinol, and MQ-W alone (shown in Fig. 16.1), K7Neo, Q3Rut, Q34'Glc, Qt7Glc, M3Rha, S3Gal, M7Gal, αG-Rut, Q5Tri, and G-Hes showed statistically significant SCC only against solutions containing silver iodide, while Q3Glc and A7Glc showed statistically significant SCC only against solutions containing INB *E. ananas* (Kuwabara et al. 2012; Table 16.2). On the other hand, while many flavonoid compounds exhibited statistically significant SCC against two INs, flavonoid compounds including K3Rut, Q7Glc, Q7Gal, Q3 (Glc) n, and P3Glc exhibited statistically significant SCC against solutions containing three INs with different degrees of SCC (°C) depending on the difference in INs (Kuwabara et al. 2012; Table 16.2). In tannin-related compounds examined against three or five INs (shown in Fig. 16.1), EC, CG, Amp3G, MG, and RA showed statistically significant SCC only against solution containing silver iodide (Kuwabara et al. 2013; Table 16.3). On the other hand, while many tannin-related compounds exhibited statistically significant SCC against two kinds of INs, tannin-related compounds including tri-GGlc, tet-GGlc, M-tet-GGlc, tri-GHam, ECG, EgaCG, and gaCG exhibited statistically significant SCC against solutions containing three INs with change in the degree of SCC (°C) depending on the difference in INs (Kuwabara et al. 2013; Table 16.3). Polyphenol mixtures and crude tannin extracts also showed a similar tendency of SCC with identified polyphenols described above on their responses to different INs (Table 16.5). It should be noted that although many supercooling-promoting polyphenols exhibit SCC to many INs, no supercooling-promoting polyphenols that exhibit statistically significant SCC to all (five kinds) INs examined have been found (Tables 16.2, 16.3, and 16.4).

SCPSs Producing INC Against Specific INs

It should be noted that some of the supercooling-promoting polyphenols (Tables 16.2 and 16.3) and some of the polyphenol mixtures and crude

tannin extracts (Table 16.5) even exhibit statistically significant INC as a negative activity of SCPSs against specific INs. INC by polyphenol compounds is restricted to MQ-W alone which contains only unidentified heterogeneous INs, and the compounds have no INC in solutions containing identified heterogeneous INs. However, SCPSs that enhance INC against identified INs have rarely been reported. A preliminary study by Holt (2003b) showed INC by AFP III from the ocean pout *M. americanus* and by AFGP from the Antarctic cod *D. mawsoni* against solutions containing INB *P. syringae*, although the magnitude of INC was low (>0.6 °C) without examining statistical significance. Some flavonoid compounds (Kuwabara et al. 2012; Table 16.2), some tannin-related compounds (Kuwabara et al. 2013; Table 16.3), and some polyphenol mixtures and crude tannin extracts (Kuwabara et al. 2014; Table 16.5), which showed statistically significant SCC against many identified heterogeneous INs, strongly enhanced INC against MQ-W alone. It is notable, however, that these SCPSs, including K7Glc, RA, and CT, which showed statistically significant INC against MQ-W alone by a droplet freezing assay (Tables 16.2 and 16.3) were shown to have no effect on homogeneous ice nucleation temperature by an emulsion freezing assay (Kuwabara et al. 2012, 2013; see Sect. 16.4.2). Therefore, it is concluded that INC of these polyphenols against MQ-W alone may result from the formation of INs by interaction of these SCPSs with impurities (unidentified heterogeneous INs) in MQ-W, but not due to INC of these polyphenols alone. INCs of other SCPSs against water containing only unidentified INs have not been reported, except for the case of an excessive concentration of AFP I from the winter flounder *Pleuronectes americanus* being used in 300 mM saline solution (Wilson et al. 2010; see Sect. 16.4.2.2).

On the other hand, an increase in the degree of SCC (°C) in solutions containing only unidentified INs has been reported for AFGPs from the Antarctic cod *D. mawsoni* in a saline solution (Wilson and Leader 1995; Table 16.1), for AFP III from winter flounder *P. americanus* in saline solution (Wilson et al. 2010), for AFGP from Antarctic cod *D. mawsoni* and for AFP III from ocean pout *M. americanus* in filtered tap water (Holt 2003b), for MMANVP in distilled water (Caple et al. 1983a), and for NMMANVP in tap water (Holt 2003b), though MQ-W was not used in those studies and the water might therefore have contained many unidentified impurities as heterogeneous INs (Table 16.1). Among identified polyphenol compounds in which many compounds exhibited INC against MQ-W alone, only tri-GGlc exhibited statistically significant SCC in MQ-W alone (Kuwabara et al. 2013; Table 16.3). Polyphenol mixtures, including S90, SEgaCG, and OLGX, exhibited also statistically significant SCC in MQ-W alone (Kuwabara et al. 2013; Table 16.5). Interactions of SCPSs with unidentified INs may be complex, showing both positive and negative effects on freezing response, probably due to the difference in compounds produced by interactions between SCPSs and INs. The INC by unidentified INs in MQ-W alone will not affect freezing temperatures of solutions containing identified heterogeneous INs because freezing of MQ-W alone by unidentified INs occurs at temperatures much lower than those in solutions containing diverse kinds of identified INs.

It has been suggested that heterogeneous INs may organize ice-like aggregations of water molecules on the surfaces of INs, by which INC is enhanced. SCPSs may preferentially bind to such active water organizing sites of INs by that INC may be inhibited or reduced (Zachariassen and Kristiansen 2000; Duman 2002; Wilson et al. 2003). SCPSs which promote supercooling against only restricted INs may have specifically matching sites of binding to water organizing sites in such INs. However, such mechanism of binding of SCPSs may be greatly different depending on characteristics of individual INs. Very recently, Inada et al. (2017) have proposed the specific binding mechanism of a supercooling-promoting surfactant HTAB 16 to silver iodide.

It is noted that many SCPSs have a tendency to be more effective against INs with a high capability to induce freezing at higher temperatures such as silver iodide and INB *E. ananas* than against INs with a lower capability to induce

freezing at lower temperatures such as INB *X. campestris*, phlorologlucinol, and MQ-W alone (compare Fig. 16.1 showing FT_{50} in solutions containing different INs and Tables 16.2, 16.3, 16.4, and 16.5 showing the SCC of SCPSs against these solutions containing different INs), although for freezing at higher subzero temperatures, SCPSs need to inhibit the effect of INs that organize ice-like aggregation from much more water molecules than those at lower temperatures (Vali 1995). Details of interactions between SCPSs and heterogeneous INs in relation to temperatures remain to be determined. The mechanisms by which SCPSs exhibit SCC against a variety of INs also remain unclear, though it has been speculated that such SCPSs may contain diverse matching sites to bind to ice nucleation sites of INs with different structures.

16.4.2.2 Effects of Different Concentrations of SCPSs

SCC (°C) changes depending on the concentrations of SCPSs, probably, in close relation to the concentrations of heterogeneous INs in solutions. Within the limited range of concentrations of SCPSs examined, a gradual increase in the magnitude of SCC (°C) with an increase in the concentration of SCPSs at a fixed concentration of INs has been shown. Wowk and Fahy (2002) showed a gradual increase of SCC in parallel with increase in PVA concentrations in the range of 0.001% to 0.1% (v/v) against solution containing INB *P. syringae* (Table 16.1). SCC of AFGP from the Antarctic cod *D. mawsoni* in tap water increased with increase in the concentration from 0.1% to 1.0% (w/v) (Holt 2003b; Table 16.1). In crude xylem extracts from several boreal tree species, SCC against a solution containing INB *E. ananas* increased with increase in concentrations of the extracts from 10 to 100 mOsml/kg (Kasuga et al. 2007). In the flavonol compounds K3Glc, K7Glc, and Q3Glc that were purified from deep supercooling xylem parenchyma cells of the katsura-tree, SCC was higher at 0.05% than at 0.01% (w/v) against several INs including INB *E. ananas*, INB *X. campestris*, silver iodide, and phlorogluc inol in solutions (Kuwabara et al. 2011). SCC of four hydrolysable tannins, tri-GGlc, tet-GGlc, pent-GGlc, and M-tet-GGlc, purified from xylem of the katsura-tree increased with increase in concentrations from 0.01 to 1.00 mg/ml against solutions containing INB *E. ananas* (Wang et al. 2012; Table 16.1). The cationic surfactant C16TAB showed a concentration-dependent linear increase of SCC from 5.7 to 11.3 °C at concentrations from 0.0005% to 0.01% (v/v) against silver iodide (Kuwabara et al. 2014). In all SCPSs reported, SCC of C16TAB was highest at the lowest concentration. The results suggested that the increase in SCC with increase in concentrations of SCPSs might indicate the presence of a much larger content of INs in solutions than the applied concentrations of SCPSs.

A limitation of the concentrations of SCPSs for increasing SCC has also been shown. Parody-Morreale et al. (1988) showed that AFGP from the Antarctic cod *D. mawsoni* decreased the INC against INB *Erwinia herbicola* up to a certain concentration, beyond which there was no further change. In a solution containing silver iodide, SCC of the nonionic surfactant E2025G increased from 4.9 to 10.7 °C with increase in concentration from 0.001% to 0.005% (v/v) but became a plateau with further increase in the concentration to 0.1% (Kuwabara et al. 2014). No further increase in SCC after a certain concentration of SCPSs suggests that the inhibitory effect of SCPSs against INs is saturated at a certain concentration and that a further increase in the concentration of SCPSs does not affect freezing anymore. A reduction of SCC by increased concentration of SCPSs over certain limit of concentrations is also shown. AFP III from ocean pout *M. americanus* showed higher SCC at 0.5% than at 1.0% against tap water (Holt 2003b). The SCC of type I AFP from the winter flounder *P. americanus* gradually increased in 500 mM NaCl solution when the concentration was increased from 1 to 8 mg/mL, but SCC decreased at higher concentrations (Wilson et al. 2010). SCC of the nonionic surfactant TX-100 against silver iodide was 3.5 °C at 0.001% (v/v) and increased to 11.5 °C at 0.1% but decreased to 9.5 °C with an increase in

the concentration to 0.5% (Kuwabara et al. 2014). SCC of PVA against filtered tap water increased with an increase in concentration from 0.1% to 0.5% (w/v) but decreased at the concentration of 1.0% (Holt 2003b; Table 16.1). The results suggest that the SCC of SCPSs increases at low concentrations but decreases with excessive concentrations of SCPSs, possibly caused by aggregation of SCPSs due to their insolubility (Wilson et al. 2010). It has also been reported that an excess concentration of SCPSs results in INC. Type I AFP from the winter flounder *P. americanus* showed INC in 300 mM NaCl solution at a concentration exceeding 20 mg/mL (Wilson et al. 2010). In MQ-W alone, Q3Glc at a concentration of 0.001% (w/v) showed SCC, but it showed INC at a concentration of 0.005% (Kuwabara et al. 2011). AFP III from the ocean pout *M. americanus* against INB *P. syringae* (Snomax) showed a slight increase in SCC (<0.5 °C) at a concentration of 0.1% (w/v) but showed INC at concentrations of 0.5–1.0% (Holt 2003b). K3Glc and K7Glc, which showed statistically significant INC against MQ-W alone, showed a gradual increase in INC with increases in their concentration from 0.001% to 0.05% (w/v) (Kuwabara et al. 2011). The results suggest that changes in response of SCPSs depending on their concentrations might be caused by their solubility, at least for some SCPSs. Our study showed that change of pH in solutions by a diluted buffer resulted in a change in the degree of SCC or INC for some SCPSs, probably due to change in the solubility of SCPSs, although the possibility of an effect of pH on the function of INs themselves cannot be ruled out (unpublished result). It is also notable that solubility of some SCPSs is gradually reduced by a reduction in temperature and produce aggregates of SCPSs which may affect to the freezing response of water.

16.4.2.3 Effect of Water Volume

In freezing of water, proportionality of nucleation probability with volume is predicted in both homogeneous and heterogeneous ice nucleation. For homogeneous nucleation, the mean freezing temperatures of different volumes will decrease linearly with the logarithm of volume reduction (Vali 1995). For heterogeneous nucleation, however, simple proportionality to volumes is often obscured by the chance allocation of heterogeneous INs (Vali 1995). Zachariassen and Hammel (1988) showed clear change of heterogeneous ice nucleation temperatures with volume using central inflorescence fluid from the Afro-alpine plant *Lobelia telekii*, which contains highly potent INs, special INBs that live in symbiosis. They examined the supercooling points of diluted central fluids of different volumes and showed reduction of supercooling points by increasing the volume of fluids. For example, the freezing points in volumes of 1000, 100, 10, and 1 μL central fluids diluted to one hundredth from the original concentration were gradually reduced from −5, −7, and −13 to −22 °C, respectively, with reduced volumes. Together with results for freezing temperatures in the central fluids diluted by a factor of 10 from 1 to 1000, they concluded that all samples with the same content of INs decided freezing temperatures regardless of the sample volume and concentrations of INs. Therefore, volume dependency of nucleation temperature is a result of change in the content of INs in both homogeneous and heterogeneous freezing.

Based on the assumption regarding the function of SCPSs, which may suppress the function of INs by binding to organizing sites of water molecules in INs, and based on the results showing dependency of SCC on the concentrations of SCPSs against solutions containing identified heterogeneous INs, it is predicted that the effect of SCPSs will not essentially change with change in the volume if the same concentration of SCPSs is added to a solution containing a fixed concentration of heterogeneous INs, regardless of the volume. Koyama et al. (2014) compared the magnitudes of SCC (°C) of several supercooling-promoting polyphenols against silver iodide in different volumes of solutions between 0.5 pL (samples for emulsion freezing assay) and 2 μL (samples for droplet freezing assay). Recently, they also compared SCCs of several surfactants against silver iodide in volumes between 0.5 pL

and 2 μL (Inada et al. 2017). Although both studies showed comparatively higher SCC in samples for emulsion freezing than in samples for droplet freezing, an exact comparison of SCC by volume is meaningless in those studies because the concentrations of silver iodide are clearly different between the water droplets for the two assays (Koyama et al. 2014; Inada et al. 2017). Therefore, we briefly compared the effects of SCPSs in different volumes of MQ-W containing several species of polyphenol compounds at a concentration of 0.1% (w/v) with the effects in solutions containing the same concentration (0.5 mM) of silver iodide. By cooling at a rate of 0.2 °C/min, SCC in 2 μL (on plate) and 1 mL (in plastic tube) solutions were 5.8 and 3.0 °C for Q3 (Glc) n, 2.9 and 1.4 °C for SEgaCG, 8.5 and 7.3 °C for TC, 4.8 and 4.1 °C for TA, and 4.7 and 4.7 °C for OLG, respectively, showing little difference in SCC regardless of volume (unpublished data). However, due to the difference of experimental conditions other than volume, a simple comparison for volume may be problematic. It has been shown that variation of SCC in bulk water may arise from not only volume but also the vessel and other conditions (Wilson et al. 2010).

16.5 Further Properties of SCPSs

16.5.1 Effect on Long-Term Stability

The capability of SCPSs is usually judged by the magnitude of SCC (°C). On the other hand, Wowk and Fahy (2002) revealed the SCC of PVA and PGL by counting the reduced number of ice crystals that developed in vitrified 55% EG solution by visual observation. Here, we examined the SCC of several SCPSs by long-term stability. In 1 mL MQ-W containing several species of SCPSs, the stability of supercooling was examined as a function of holding time at −10 °C under a static condition (Fig. 16.2). MQ-W alone was gradually frozen with prolonged time, and 50% of the samples were frozen within 1 week. Compared with MQ-W alone, solutions containing 0.1% NaCl, which have no SCC, had improved the stability of

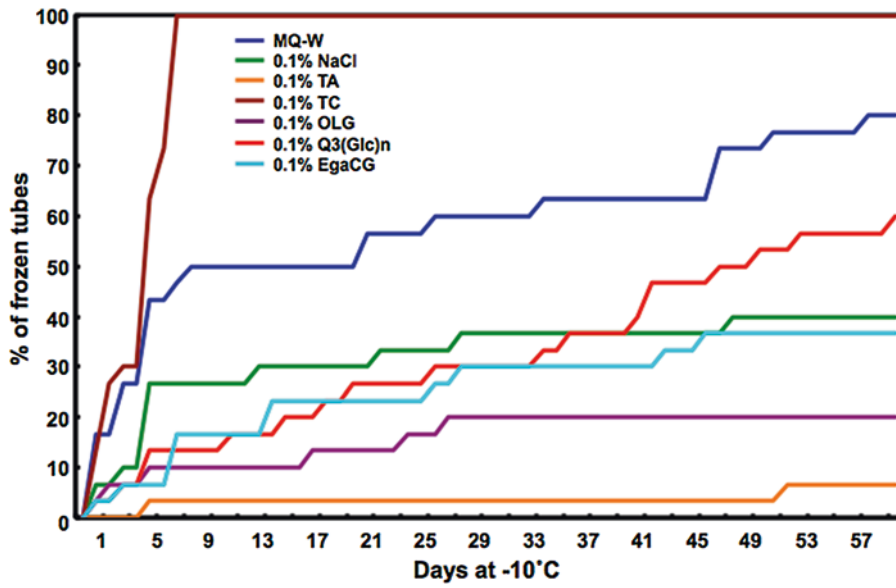

Fig. 16.2 Long-term (days) holding effect of supercooling stability in 1 mL MQ-W containing NaCl or several species of SCPSs under a static condition at −10 °C. Ratio of frozen tubes was calculated from $n = 30$ in each sample. *MQ-W* MQ-water alone for control, *TA* tannic acid, *TC* tea catechin, *OLG* Oligonol, *Q3(Glc)n* α-oligoglucosyl quercetin 3-*O*-β-D-glucopyranoside, *EgaCG* (−)-epigallocatechin gallate

supercooling for long-term holding with less than 50% of the samples being frozen after holding for more than 40 days. While many SCPSs, including Q3 (Glc) n, TA, EgaCG, and OLG, exhibited similar supercooling stability in long-term holding with NaCl solution, some SCPSs showed different responses. One mL MQ-W containing 0.1% TC is completely frozen within 5 days much earlier than the freezing time for MQ-W alone. Droplet freezing showed that TC in MQ-W had INC of −5.3 °C (Table 16.5), indicating that the freezing of droplets containing TC occurred at around −17 °C in droplet freezing (FT_{50} of control MQ-W alone being around −22 °C; see Fig. 16.1). It was shown that although the freezing temperature of TC solution by droplet freezing is −17 °C, 1 mL TC solution was frozen at a much higher temperature (−10 °C) by long-term holding. It is unclear whether most SCPSs which showed INC in droplet freezing assay show similar tendency with TC by long-term holding or not. On the other hand, 1 mL TA solution showed good stability against long-term static holding at −10 °C, with less than 5% of the samples being frozen after holding for 60 days. Although in droplet freezing, not only TA but also Q3 (Glc) n, EgaCG, and OLG in MQ-W did not affect freezing temperatures (Table 16.5), a strong stabilizing effect of supercooling for long-term holding at −10 °C is found only in TA. The results suggest that the SCC of specific SCPSs such as TA for stabilizing supercooling state for long term may arise from additional mechanisms different from the general capability of SCPSs to increase the degree of SCC (°C).

16.5.2 Effect on Mechanical Disturbance

There are well-known observations related to the freezing of supercooled bulk water by mechanical disturbance (Vali 1995). Water in a bottle in a static condition is often supercooled to a subzero temperature even near −10 °C, but freezing takes place spontaneously soon after pouring the supercooled water into a cup. Such freezing occurs due to mechanical disturbance of supercooling, particularly by formation of air bubbles in water. Inada et al. (2001) showed that ultrasonic-induced cavitation in water promoted freezing of MQ-W and tap water which are filled in vessels with an inner diameter of 140 mm, in warmer temperature about 8 °C higher than freezing temperature in static conditions. Heneghan and Haymet (2002) showed that bubbling of 200 μL distilled water in a glass tube caused the ice nucleation temperature to increase by approximately 2.4 °C compared with that in water without bubbles. They suggested that a bubble is a surface on which nucleation may occur by reducing the effective free energy barrier to nucleation.

Since, as far as we know, there has been no study on the effects of SCPSs with mechanical disturbance of an aqueous solution, we examined the effects of SCPSs under the condition of mechanical disturbance of 1 mL MQ-W containing several SCPSs, including SEgaCG, S90, OLG, and TX-100, in plastic tubes overnight at different subzero temperatures (unpublished results). The results showed that the stability of supercooling against mechanical disturbance changed greatly depending on the species of SCPSs (Fig. 16.3). In 1 mL of MQ-W alone (concentration 0), all of the tubes were completely frozen at −5 °C within 24 h under the condition of strong shaking. In MQ-W containing SEgaCG, strong shaking caused almost complete freezing of all samples even at −5 °C at a concentration of 0.1%. On the other hand, S90, OLG, and TX-100 showed a reduction of the freezing ratio under the condition of mechanical disturbance, though the ratio of stability changed depending on the species of SCPS, temperature, and concentration of SCPSs. Under the condition of the strong shaking, the freezing ratios of the SCPSs increased gradually with reduction of temperature from −5 to −10 °C, and the freezing ratios were decreased by an increase in the concentrations of SCPSs from 0.01% to 0.1%. Among the SCPSs examined under the condition of mechanical disturbance, TX-100 at a concentration of 0.1% showed the greatest stability, with supercooling being maintained at −10 °C in almost 80% of the

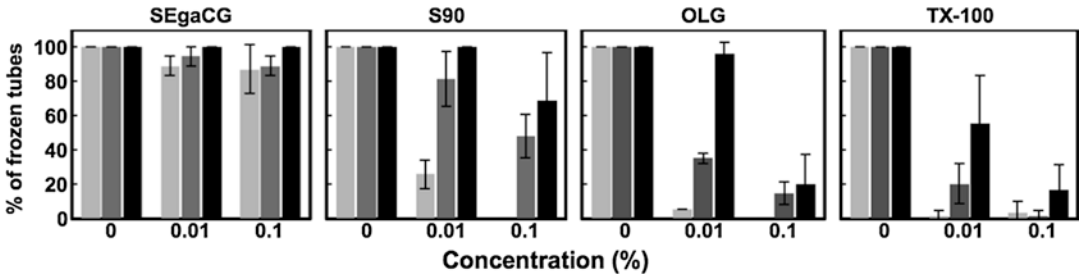

Fig. 16.3 Effect of mechanical disturbance on supercooling stability of MQ-W containing different concentrations of SEgaCG, S90, OLG, and TX-100. One mL solution in a plastic tube was kept at −5 (gray), −7.5 (dark-gray), and −10 °C (black) for 24 h under the condition of strong shaking at 2500 rpm. Ratio of frozen tubes was calculated from $n = 54$ in each sample. *SEgaCG* Sunphenon epigallocatechin gallate, *S90* Sunphenon 90S, *OLG* Oligonol, *TX-100* triton X-100

samples. The degree of SCC (°C) of those SCPSs against MQ-W alone as examined by droplet freezing did not correspond to the difference in stability of the SCPSs against mechanical disturbance. Although the results showed that stability against mechanical disturbance was lowest for SEgaCG and highest for TX-100, the SCC of these two SCPSs was not affected in MQ-W alone as measured by droplet freezing (Tables 16.4 and 16.5). Although the stability of OLG against mechanical disturbance was higher than that of S90, droplet freezing showed that S90 had statistically significant SCC in MQ-W alone (Table 16.5), whereas there was no change in SCC of OLG against MQ-water alone. Therefore, the stability of specific SCPSs such as TX-100 and OLG against mechanical disturbance may arise from different mechanisms with increased degree of SCC as well as long-term stability of supercooling.

16.5.3 Effect on Interactions of SCPSs in the Presence of Solutes

The SCC of SCPSs may be enhanced by the combination or interaction of multiple SCPSs. An increase in SCC by the combination of SCPSs was shown by Wowk and Fahy (2002). They showed that the inhibitory effect on ice nucleation in a 55% EG solution containing INB *P. syringae* became greater with the addition of both PVA and PGL than with the addition of PVA or PGL alone. Furthermore, higher SCC (°C) and extensive effect against various INs were seen in supercooling-promoting polyphenol mixtures and crude tannin extracts (Kuwabara et al. 2013; Table 16.5) compared to the effects of identified single tannin-related compounds (Kuwabara et al. 2013; Table 16.3). For example, polyphenol mixtures and crude tannin extracts against INB *E. ananas* and silver iodide showed statistically significant SCC in all mixtures and extracts examined (Table 16.5), while some of the identified tannin-related compounds against the same INs showed no SCC (Table 16.3). The presence of statistically significant SCC against MQ-W alone is also a characteristic of many polyphenol mixtures (Kuwabara et al. 2013; Table 16.5). It is thought that the presence of many kinds of SCPS in polyphenol mixtures and crude tannin extracts may promote SCC against various INs by their combination or interaction effects.

Very interesting results obtained by Duman's group (Olsen and Duman 1997; Li et al. 1998; Duman 2002) showed that specific interactions of SCPS with solutes enhanced SCC. They showed that larvae of the beetle *D. canadensis* accumulate potent D-AFPs in their hemolymph and gut. The purified D-AFPs inhibited the activity of bacterial and hemolymph protein INs, but the inhibitory effect was not so strong with SCC of 1.7 °C against protein INs and SCC of 1.2 °C against INB *P. syringae* (Table 16.1). However, in the presence of 1 M glycerol, the SCC of D-AFPs increased to 7.8 °C against protein INs and to 9.4 °C against INB *P. syringae*. Furthermore, in the presence of 0.5 M citrate,

SCC of the D-AFPs increased to 6.9 °C against protein INs and to 13.7 °C against INB *P syringae*. It should be noted that although the addition of 1 M glycerol or 0.5 M citrate reduced the freezing point to 2–6 °C by equilibrium freezing temperature depression and accompanying supercooling, there are clear specific enhancing effects of glycerol and citrate on the SCC of D-AFPs. Enhancement of the SCC of D-AFPs by the presence of certain proteins has also been reported (Wu and Duman 1991). The mechanism by which inhibitory effect of INs is enhanced by these solutes has been speculated to be increased binding of D-AFPs to INs based on the solute exclusion model proposed by Timasheff (1992) and Duman (2002).

Our preliminary study that was carried out to test effects of SCPSs in order to promote SCC in various kinds of aqueous solutions, such as incubation media for medical purposes, beverages and beers by the addition of diluted SCPSs showed large variations in the magnitude of SCC (°C) by addition of the same SCPS in different aqueous solutions. Since it was speculated that the difference in SCC might arise from interactions of SCPSs with different solutes contained in different aqueous solutions, we investigated changes in SCC by interaction of several substances such as interactions between randomly chosen SCPSs in the presence of different kinds of electrolytes and sugars. The results showed a large variation of SCC depending on the combination of SCPSs in the presence of different solutes (unpublished results). For example, one of the effective combinations that improved long-term stability of supercooling was obtained by interaction of S90 and OLG in the presence of $CaCl_2$ and Glc (Fig. 16.4). Under a static condition at −20 °C, 1 mL MQ-W containing 1 M Glc + 1 M $CaCl_2$ (4 Osm) showed freezing of 40% samples within 1 day, gradual increase in the freezing ratio by prolonged holding, and almost complete freezing of the samples after 80 days. The addition of either 0.5% S90 (12 mOsm) or 0.1% OLG (0.8 mOsm) to 1 M Glc + 1 M $CaCl_2$ solution improved the long-term stability of supercooling, with about 80% of

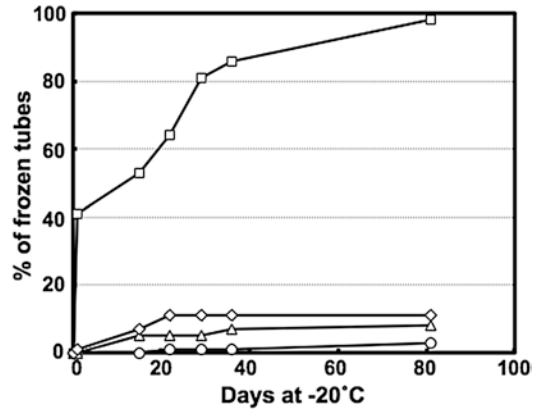

Fig. 16.4 Long-term (days) holding effect of supercooling stability in 1 mL MQ-W containing 1 M Glc + 1 M $CaCl_2$ (square), 1 M Glc + 1 M $CaCl_2$ + 0.5% S90 (diamond), 1 M Glc + 1 M $CaCl_2$ + 0.1% OLG (triangle), and 1 M Glc + 1 M $CaCl_2$ + 0.5% S90 + 0.1%GLC (circle) under a static condition at −20 °C. Ratio of frozen tubes was calculated from n = 100 in each sample. *S90* Sunphenon 90S, *OLG* Oligonol

unfrozen samples after holding for 80 days, in each sample. The greatest improvement in long-term stability of supercooling was achieved when both 0.5% S90 and 0.1% OLG were added together in 1 M Glc + 1 M $CaCl_2$ solution, showing almost complete stability of supercooling at −20 °C for 80 days. The results suggested that interaction of supercooling-promoting S90 and OLG in the presence of Glc and $CaCl_2$ produced higher stability of supercooling against long-term holding at subzero temperatures.

Examination using the abovementioned combination of SCPSs and solutes in 1 mL MQ-W also showed high stability of supercooling against mechanical disturbance (Fig. 16.5). Under the condition of strong shaking of 1 mL solution for 24 h at −20 °C, 1 M Glc or 1 M $CaCl_2$ solutions were completely frozen in a manner similar to that with MQ-W alone. More than 70% of the solutions containing only 0.5% S90 or 0.1% OLG as well as both 0.5% S90 and 0.1% OLG were also frozen. The freezing rate of a solution containing 1 M Glc + 1 M $CaCl_2$ was reduced to about 25% by their high Osm. While combinations lacking one of the solutes did not prevent freezing, even though the freezing ratio

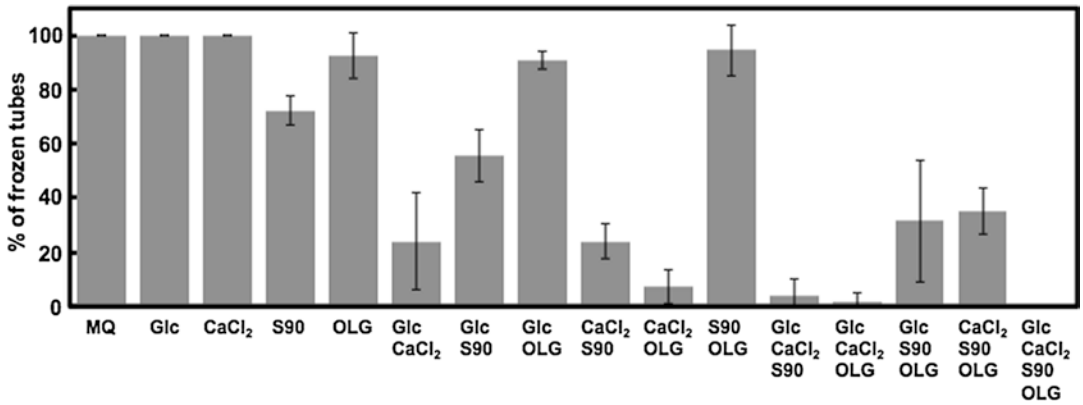

Fig. 16.5 Effect of mechanical disturbance on supercooling stability by interactions of supercooling-promoting S90 and OLG with Glc and $CaCl_2$. Tubes each with 1 mL MQ-W containing different chemicals were kept at −20 °C for 24 h under the condition of strong shaking at 2500 rpm. The concentrations were Glc = 1 M, $CaCl_2$ = 1 M, S90 = 0.5%, and OLG = 0.1%. Ratio of frozen tubes was calculated from n = 100 tubes in each sample. *S90* Sunphenon 90S, *OLG* Oligonol

was very low, the combination of four solutes, 1 M Glc + 1 M $CaCl_2$, 0.5% S90, and 0.1% OLG, completely prevented freezing at −20 °C under the condition of strong shaking overnight. In this connection, supercooling points of these solutions when 1 mL solutions are cooled at a cooling rate of 0.2 °C/min showed freezing points of −27.3 ± 2.1 °C (n = 20) in 1 M Glc + 1 M $CaCl_2$ and −27.2 ± 3.7 °C in 1 M Glc + 1 M $CaCl_2$ + 0.5% S90 + 0.1% OLG (n = 20) without statistical difference. All of these results indicate potent improvement of SCC for long-term holding as well as for mechanical disturbance by specific interactions of SCPSs with other solutes. Further study on these interactions to enhance specific SCC may be necessary for useful applications of SCPSs.

16.5.4 Effect on Ice Crystals

While AFPs and AFGPs can inhibit ice crystal growth by binding to nascent ice crystals in a temperature range called thermal hysteresis, many of them also have the ability to increase SCC by binding to INs (Table 16.1). Although the effects of ice crystal binding and IN binding may be different (Duman 2002), it is unclear whether SCPSs other than AFPs and AFGPs have an ice crystal binding effect or not. Therefore, we examined the ice-binding effects of randomly chosen SCPSs including supercooling-promoting flavonoid compounds such as Q3 (Glc) n and αG-Rut, tannin-related compounds such as C, EC, EgaC, EgaCG, and Pent-GGlc; polyphenol mixtures and crude tannin extracts such as SEgaCG, TC, TA, S90, OLG, and LFP; and surfactants such as C16TAB and TX-100 (Fig. 16.6) using a microscopy for examining the ice-binding effects of AFPs (Kobashigawa et al. 2005). The results showed that while AFP II from the Japanese pond smelt *Hypomesus nipponensis* had ice-binding capability producing bipyramid-shaped ice crystals, all of the SCPSs examined lacked capability to inhibit ice crystal growth, producing round-shaped ice crystals similar to ice crystals produced by usual water freezing. It is concluded that SCC is produced by binding of SCPSs to INs without a binding effect on growing ice crystals.

We also examined the effect of SCPSs on ice sublimation using ice disks containing randomly chosen SCPSs and other reference substances without SCC (Fig. 16.7). In frozen disks at −15 °C, it was noted that while MQ-W alone showed distinct separation between ice crystals and concentrated solutes, other disks containing solutes showed a comparatively dispersed (well

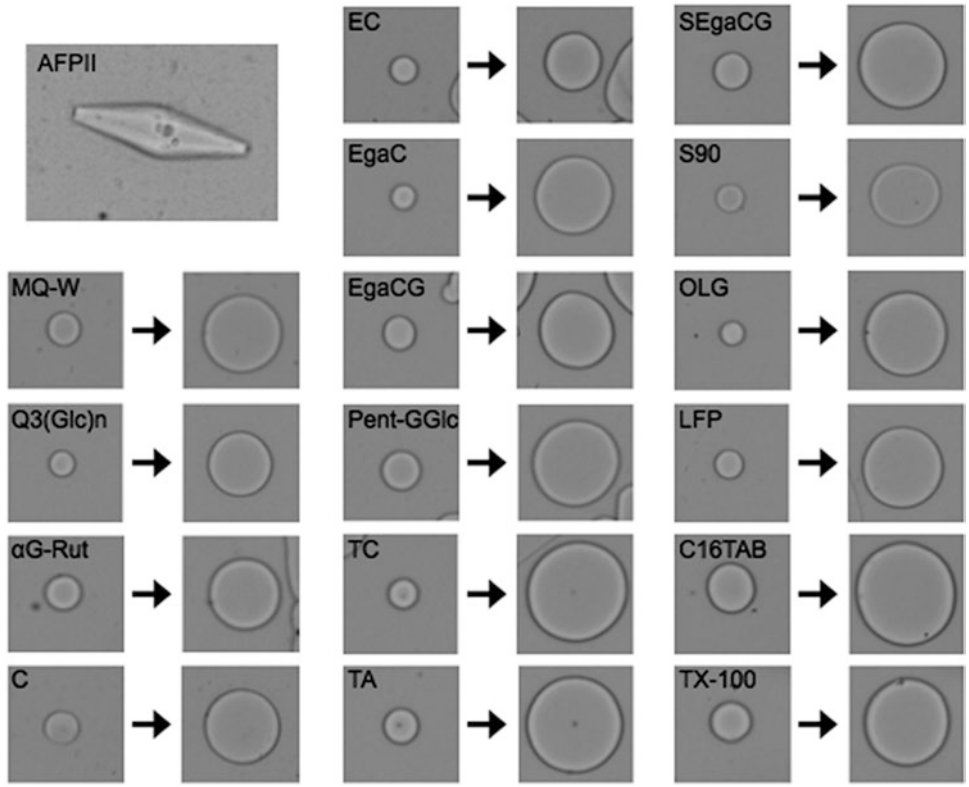

Fig. 16.6 Microscopic observation of the ice-binding effect of SCPS. Samples each of 2 μL of MQ-W containing various SCPSs (0.1 w/v %) were frozen and thawed to near 0 °C until a partially frozen state in which liquid and small ice crystals were mixed (left side). They were cooled at 0.4 °C/min, and the growth of ice crystals was observed (right side). Ice crystal growth of SCPSs is compared with that in AFP II from the Japanese pond smelt *Hypomesus nipponensis*, which has an ice-binding effect. *AFP II* antifreeze protein II, *MQ-W* MQ water alone for control, *Q3(Glc)n* α-oligoglucosyl quercetin 3-*O*-β-D-glucopyranoside, *αG-Rut* 4^G-α-D-glucopyranosyl rutin, *C* (+)-catechin, *EC* (−)-epicatechin, *EgaC* (−)-epigallocatechin, *EgaCG* (−)-epigallocatechin gallate, *Pent-GGlc* 1,2,3,4,6-penta-*O*-galloyl-α and ß-D-glucopyranose, *TC* tea catechin, *TA* tannic acid, *SEgaCG* Sunphenon epigallocatechin gallate, *S90* Sunphenon 90S, *OLG* Oligonol, *LFP* litchee fruit polyphenol, *C16TAB* hexadecyltrimethylammonium bromide, *TX-100* triton X-100

mixed) state of ice and concentrated solutes. Supercooling-promoting OLG, S90, and TA exhibited mixed state that was similar to or more mixed than that by ice-binding AFP III from the Notched-fin eelpout *Zoarces elongates*, although the reason remains unclear (Yamanouchi et al. 2013). When these ice disks were held at −15 °C for a long time, ice disks of MQ-W alone and ice disks containing Glc and trehalose (Tre) were gradually sublimated with time, and most of the ice crystals had disappeared by sublimation, leaving only concentrated solutes after keeping for 35 days, whereas most of the ice disks containing SCPSs including OLG, S90, TA, and AFP III were maintained after holding for 35 days due to retarded sublimation. Although Tre has high affinity to bind water molecules (Sakurai 2012), non-supercooling-promoting Tre does not retard ice sublimation. It was also noted that stability against sublimation appeared to be higher in OLG, S90, and TA, which have no ice-binding effect, than in AFP III, which has an ice-binding effect. It is interesting that while SCPSs do not bind to growing ice crystals (Fig. 16.6), they

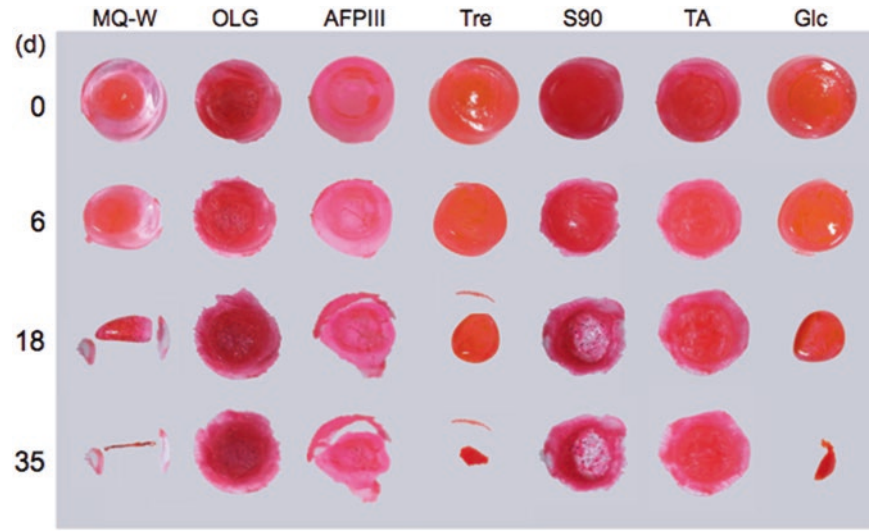

Fig. 16.7 Effect of SCPSs on sublimation of ice disks. Samples each of 10 mL MQ-W containing indicated substances (for SCPSs, OLG, AFP III, S90, and TA; for control MQ-W alone, Tre and Glc) at a concentration of 0.1% (w/v) with red ink (0.3% v/v) were frozen in glass cups of 3 cm in diameter and 2 cm in height at −15 °C overnight. The ice disks were removed from the cups, placed on paper plates, and kept at −15 °C for the indicated days (d) to allow sublimation. *OLG* Oligonol, *AFP III* antifreeze protein III from the Notched-fin eelpout *Zoarces elongates, Tre* trehalose, *S90* Sunphenon 90S, *TA* tannic acid, *Glc* glucose

retard the sublimation of ice crystals, suggesting the presence of an unknown interaction of SCPSs with ice crystals. The mechanism by which these SCPSs inhibit ice sublimation is unclear, but this special phenomenon might be important to understand the functions of SCPSs.

16.6 Outlook

The biological importance of SCPSs has been gradually recognized, but there have not been sufficient studies. For successful overwintering, freeze-avoiding insects accumulate supercooling-promoting AFPs to inhibit freezing by INs located in body fluids. The successful inhibition of the effect of INs in insects is achieved by enhancement of the SCC of AFPs by interaction with accumulated proteins (Wu and Duman 1991) and glycerol during winter (Duman 2002). Although the presence of SCPSs other than AFPs and AFGPs in overwintering insects has not been reported, the possibility of their existence should be examined to understand overwintering mechanisms in a wide variety of insects. Xylem parenchyma cells in trees adapt by deep supercooling to avoid the effect of freezing. Boreal trees can adapt successfully to winter cold by seasonal changes of SCC in xylem cells below environmental temperatures from around −10 °C in warmer seasons to below −40 °C during cold winter (Fujikawa et al. 2009). Supercooling-promoting polyphenols have been discovered in the xylem cells of the trees (Kasuga et al. 2008; Wang et al. 2012). The exact roles of these SCPSs in seasonal fluctuation of SCC in xylem cells are still unclear, although it has been suggested that interactions of supercooling-promoting polyphenols with intracellular solutes may be responsible for the seasonal fluctuation of SCC (Fujikawa 2016). In a common adaptation mechanism of cells in most plants by extracellular freezing, although many flavonoids, some of which correspond to SCPSs, are accumulated in parallel with increased freezing tolerance in different *Arabidopsis thaliana* genotypes, their exact roles

in freezing tolerance remain to be determined (Corn et al. 2008).

Studies on applications of SCPSs have also been carried out, though the range of applications is currently limited to only a few fields. Fahy's group has continued to examine the usefulness of supercooling-promoting PVP and PGL for successful preservation of animal organs by vitrification (Wowk et al. 2000; Wowk and Fahy 2002). Commercially sold chemicals, modified PVP and PGL, have been examined for vitrification preservation of mouse embryos in order to prevent ice crystal formation during warming by devitrification (Badrzadeh et al. 2010). The usefulness of a supercooling-promoting flavonol glycoside, K7G, for preservation of shoot apices of cranberry by a diluted vitrification solution has been examined in order to reduce the chemical toxicity by the original high concentration of vitrification solution (Kami et al. 2008). The usefulness of K7G for supercooling preservation of a rat heart has also been examined (Shimada et al. 2010). The usefulness of a non-metabolizable glucose derivative, 3-*O*-methyl-D-glucose, for supercooling preservation of a rat liver has also been examined, though SCC of this glucose alone has not been shown (Berendsen et al. 2014).

As stated in this review, there have not been systematic studies on SCPSs. Most published reports have provided only phenomenological information such as identification of SCPSs and the magnitude of SCC (°C) against heterogeneous INs. In this review, further characteristics of SCPS such as stabilizing effects on a supercooling state for a long term as well as stabilizing effects of some SCPSs against mechanical disturbance were introduced. Enhancement of SCC by interactions of SCPSs in the presence of specific solutes was also shown. It was noted, however, that the mechanisms underlying these properties of specific SCPSs are unknown. Furthermore, the inhibitory effect of SCPSs on sublimation of ice crystals is mysterious. Therefore, more studies are needed to understand the mechanisms underlying the various functions of SCPSs, by which the role of SCPSs in biological overwintering may be clearer and real-world applications of SCPSs may be possible. The study of SCPSs is just starting.

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17 Applications of Antifreeze Proteins: Practical Use of the Quality Products from Japanese Fishes

Sheikh Mahatabuddin and Sakae Tsuda

Abstract

Numerous embryonic ice crystals are generated in water at the moment of freezing. These crystals grow and merge together to form an ice block that can be generally observed. Antifreeze protein (AFP) is capable of binding to the embryonic ice crystals, inhibiting such an ice block formation. Fish-derived AFP additionally binds to membrane lipid bilayers to prolong the lifetime of cells. These unique abilities of AFP have been studied extensively for the development of advanced techniques, such as ice recrystallization inhibitors, freeze-tolerant gels, cell preservation fluids, and high-porosity ceramics, for which mass-preparation method of the quality product of AFP utilizing fish muscle homogenates made a significant contribution. In this chapter, we present both fundamental and advanced information of fish AFPs that have been especially discovered from mid-latitude sea area, which will provide a hint to develop more advanced techniques applicable in both medical and industrial fields.

Keywords

Antifreeze protein · Ice-binding protein · Ice growth inhibition · Membrane protection · Thermal hysteresis · Ice recrystallization inhibition · Freeze concentration inhibition · Cell cryopreservation · Cell hypothermic preservation · Porous material fabrication

Abbreviations

AFGP	Antifreeze glycoprotein
AFP	Antifreeze protein
CTLD	C-type lectin-like domain
EC	Euro-Collins
FBS	Fetal bovine serum
FIPA	Fluorescence-based ice plane affinity
IRI	Ice recrystallization inhibition
PBS	Phosphate-buffered saline

17.1 Introduction

A typical ice block is composed of numerous single ice crystals that are created in water during freezing (Hobbs 1974). The crystals grow and merge together to form an ice block if the temperature remains below 0 °C. Antifreeze protein

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(AFP) accumulates on the surfaces of embryonic ice crystals to inhibit their growth and merging, resulting in an aggregate of tiny ice crystals instead of an ice block (Nishimiya et al. 2008a, b; Yarely and Ramos 2010a). This function of AFP may be useful for the preservation of a variety of water-containing materials such as processed foods, soups, ice creams, noodles, breads, vegetables, seeds, drinks, alcohol, medicines, cosmetics, gels, cells, tissues, and organs. In keeping the size of each ice crystal to a minimum, AFP use may greatly improve the effectiveness of preservation. In addition, fish-derived AFPs bind to the lipid bilayer to prolong the lifetime of cells under hypothermic condition (+4 °C), a function that may be applicable to short-term cell preservation techniques (Rubinsky et al. 1990, 1991, 2010). The following review of AFP structure and function describes AFP species with proven performance, the mechanisms underlying their ice- and membrane-binding functions, and recent findings enabling unprecedented AFP technologies.

17.2 AFPs for Practical Use

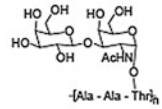
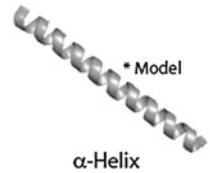
17.2.1 Fish-Derived AFGP and AFP

AFPs have been identified in the body fluid of fishes, insects, plants, and various microorganisms living in subzero temperature environments (Fletcher et al. 2001; Harding et al. 2003; Scotter et al. 2006; Davies 2014). These proteins are categorized into numerous groups based on their primary to tertiary structures, indicating the availability of numerous types of AFPs suitable for different applications. The fish-derived AFPs, for example, are categorized into antifreeze glycoprotein and type I–IV AFPs. For readability, they are occasionally denoted as AFGP and AFPI−III throughout in this chapter. Type II AFPs are subdivided into Ca^{2+}-dependent and -independent groups and type III AFPs into quaternary aminoethyl (QAE)-Sephadex- and sulfopropyl (SP)-Sephadex-binding groups (Hew et al. 1988). Artificially designed AFPs will also be regarded as a member of the group (Deluca et al. 1996; Gibson 2010). Many parameters are considered in determining the best AFP for a particular use, including the extent of ice growth inhibition, ice-shaping ability, water solubility, heat stability, acid/alkaline tolerance, molecular size, structural rigidity, and toxicity. The method of manufacture of a particular AFP, such as extraction from natural resources, production using gene technology, or solid-phase synthesis, should also be considered, as the production method is a key determinant of the product price. Another important consideration is that naturally occurring AFP is a mixture of 8–13 isoforms that function together far more effectively than any single isoform (Nishimiya et al. 2005; Wang and Duman 2005). AFGP and type I–III AFPs are the naturally occurring AFPs, for which a mass-preparation method utilizing fish muscle homogenates as the source material has been established (Nishimiya et al. 2008a, b; Mahatabuddin et al. 2017). This review focuses on selected fish-derived AFPs, describing their function and applicability (Fig. 17.1).

17.2.2 AFGP

Arthur DeVries and his colleagues first discovered AFGP in the blood serum of Antarctic notothenioids (DeVries and Wohlschlag 1969). A total of eight major fractions of AFGP have been identified, and the protein in each fraction consists of a different number of repeating units (n) of Ala-Ala-Thr, where Cβ of Thr is glycosylated with the disaccharide β-D galactosyl-(1→3)-α-*N*-acetyl-D-galactosamine. The molecular weight (MW) range of these proteins is from 2.6 kDa ($n = 4$) to 33.7 kDa ($n = 50$), where AFGP1 and 8 are the largest and smallest molecules, respectively. The concentration dependence of thermal hysteresis (TH), a measure of ice-binding strength, was examined for AFGP1–5, and the maximum TH at 1.2 °C was reported at a concentration of 40 mg/mL. This TH is approximately 20 times greater, on a molar basis, than that of AFGP8. The repeated sequence of smaller AFGPs is often substituted with different amino acids, such as Pro and Arg. Ice-etching experiments have shown that AFGP 7 and 8 bind to the prism planes of ice crystals at a concentration of 2 mg/mL (Burcham et al. 1986b; Knight et al. 1993; Harding et al. 2003).

A

Characteristic	AFGP	AFPI	AFPII	AFPIII
Typical Fish Source	 Emerald Rockcod *Notothenioidei, Gadidae*	 Winter Flounder *Pleuronectidae, Sculpin*	 Atlantic Herring *Clupeidae, Osmeridae*	 Ocean Pout *Zoarcidae*
Sequence Properties	 -[Ala - Ala - Thr]$_n$	Ala-rich, 11-res. repeats	Cys-rich, C-type lectine-like	Unbiassed, SP and QAE-groups
Mass (Da)	2.6 - 34 kDa	3.3 kDa	14 kDa	7 kDa
Structure	N/A Polyproline II helix	 * Model α-Helix	 disulfide bonded	 β-Sandwich

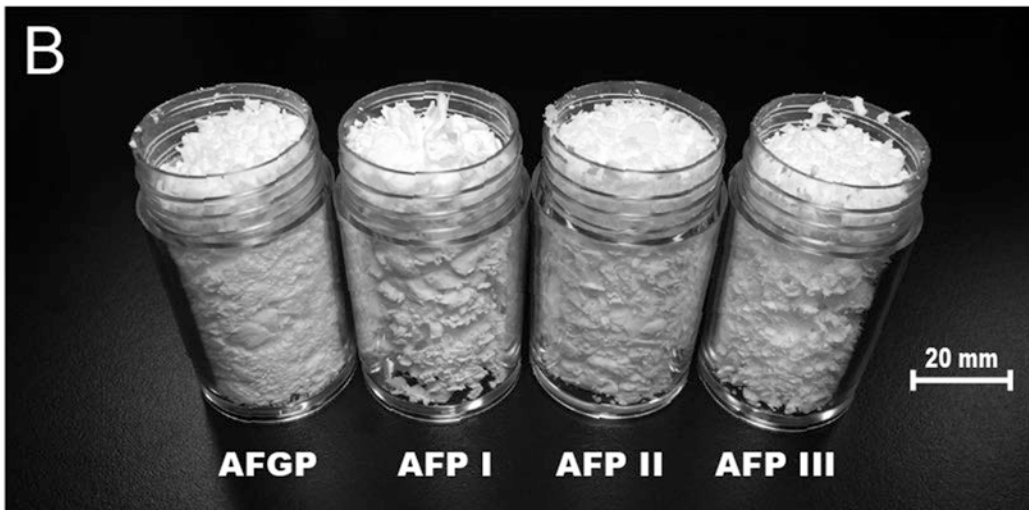


Fig. 17.1 (**a**) Types and properties of fish-derived AFGP and AFPs. The applicability of these four types of AFP has been examined extensively in both medical and industrial fields. (**b**) New AFP samples of AFPI–III and AFGP (left → right) successfully mass-purified from fish muscle (purity >95%). These samples contain neither salts nor buffer detergents and were sterilized using a 0.22 μm syringe filter

These eight AFGPs also have been isolated from Northern gadids, including the Greenland cod (*Gadus ogac*), Arctic cod (*Boreogadus saida*), and Atlantic cod (*Gadus morhua*). The saffron cod (*Eleginus gracilis*) from the Bering Sea contains a minimum of 18 fractions of Arg-containing AFGPs, differing substantially from the typical AFGPs. Several methods have been developed for creating synthetic AFGP molecules. Using multidimensional NMR spectroscopy, the structure of synthetic AFGP was determined to be an amphipathic poly-proline II-like left-handed helix from which the disaccharide moieties protrude on the same side to form a hydrophilic face (Burcham et al. 1986a, b; Tachibana et al. 2004; Gibson 2010).

17.2.3 Type I AFP

Type I AFP was first purified in 1976 from the winter flounder *Pseudopleuronectes americanus*, a native of the western coastal waters of the North Atlantic (Duman and DeVries 1976). The winter flounder-derived AFP (wfAFP) is a mixture of seven isoforms denoted HPLC1–7, in which the sixth isoform HPLC6 was the first AFP for which the crystal structure was determined (Sicheri and Yang 1995). It revealed that this 37-amino acid protein (MW, 3.3 kDa) forms an amphipathic α-helix composed of 3 repeats of the 11-residue consensus sequence (Thr-X_{10}), where X is mostly Ala. The four Thr residues are located 16.5 Å apart on one side of the helix, a distance similar to the 16.7 Å oxygen atom spacing in the pyramidal plane (20–21), the target ice plane of the winter flounder AFP identified by ice-etching experiments (Knight et al. 1993). A hydrogen-bonding network with two tightly bound water molecules on the sequence D^1TASD^5 composes the N-terminal cap structure. Another cap is formed by three hydrogen bonds contributed by the side-chain of Arg^{37} and the C-terminal NH-group. These properties were also identified in proteins of this class found in the yellowtail flounder (*Limanda ferruginea*), Alaskan plaice (*Pleuronectes quadrituberculatus*), and barfin plaice (*Liposetta pinnifasciata*) (Scott et al. 1987; Knight et al. 1993; Mahatabuddin et al. 2017). Type I AFP variants in which the N-terminal region contains α-helix-destabilizing residue (Pro or Gly) were isolated from the shorthorn sculpin (*Myoxocephalus scorpius*), grubby sculpin (*Myoxocephalus aenaeus*), and the Arctic sculpin (*Myoxocephalus scorpioides*) (Harding et al. 1999).

17.2.4 Type II AFP

Type II AFP was first isolated in 1981 from sea raven (*Hemitripterus americanus*), an inhabitant of the same seawaters as the winter flounder (Slaughter et al. 1981). This protein family was further isolated from the rainbow smelt (*Osmerus mordax*), Atlantic herring (*Clupea harengus*), Japanese smelt (*Hypomesus nipponensis*), and longsnout poacher (*Brachyopsis rostratus*) (Ewart and Fletcher 1990; Yamashita et al. 2003; Nishimiya et al. 2008a, b). The MW range for the type II AFP family is 14–24 kDa. These proteins commonly exhibit high sequence identity with C-type (Ca^{2+}-dependent) lectin-like domains (CTLDs) and contain 10 Cys residues that form five disulfide bridges. Type II AFPs are categorized into two subgroups based on the dependence of their ice-binding activity on Ca^{2+}. However, the crystal structures of the Ca^{2+}-dependent (Atlantic herring) and Ca^{2+}-independent species (longsnout poacher) have high identity. Their principle constituents are eight antiparallel β-strands associated with two α-helices to form an elongated globular structure that is highly similar to that of CTLDs, lithostathine (PDB, 1qdd), and mannose-binding protein (PDB, 1sl6). In Ca^{2+}-dependent type II AFP, the residues Asp, Thr, and Glu of the Ca^{2+}-binding loop and 1 mol of Ca^{2+} ions are assumed to bind to the first prism plane. In Ca^{2+}-independent type II AFP, the ice-binding site is a flat hydrophobic surface surrounded by a similar loop region (Gronwald et al. 1998; Liu et al. 2007).

17.2.5 Type III AFP

Type III AFP was first isolated in 1985 from ocean pout (*Macrozoarces americanus*) living in the Newfoundland coastal waters (Li et al. 1985). This AFP consists of 12 isoforms denoted HPLC1–12 (MW, 6.5–7.0 kDa). These proteins are encoded by multiple genes and are present in blood plasma during the winter at a concentration of 25–30 mg/mL (Hew et al. 1988). Proteins in the same family were extracted from Antarctic eelpout (*Austrolycicthys brachycephalus*), European eelpout (*Zoarces viviparus*), and notched-fin eelpout (*Zoarces elongatus* Kner) (Cheng and DeVries 1989; Sørensen and Ramløv 2002; Nishimiya et al. 2005). Type III AFP isoforms are categorized into QAE-Sephadex- and SP-Sephadex-binding groups that share approximately 55% sequence identity; the identity within each group is 90% and 75%, respectively. The QAE isoforms are subdivided into QAE1 and QAE2 isoforms, with only the QAE1 iso-

forms (e.g., HPLC12) capable of preventing ice crystal growth. The SP isoform only retards ice growth, becoming fully active by the addition of only a small amount (1%) of the QAE1 isoform (Takamichi et al. 2009). The structure of HPLC12 and its mutant forms have been studied extensively, revealing a slightly elongated globular shape composed of two antiparallel triple-strand β-sheets (Sönnichsen et al. 1993; Antson et al. 2001). The location of a compound ice-binding site was identified and is composed of two adjacent planar surfaces inclined at an angle of 150°, one of which binds to the ice pyramidal plane and the other to the first prism plane (Garnham et al. 2010).

17.2.6 Genera and Species of AFP-Containing Fish

AFP has been found in the muscle homogenates of many fishes living in the water areas in Japan, including those of the genus *Myoxocephalus*, *Gymnocanthus*, *Hemilepidotus*, *Furcina*, *Hypomesus*, *Spirinchus*, *Mallotus*, *Pleurogrammus*, *Sebastes*, *Clupea*, *Limanda*, *Liopsetta*, *Clidoderma*, *Cleisthenes*, *Microstomus*, *Lepidopsetta*, *Platichthys*, *Kareius*, *Eopsetta*, *Gadus*, *Theragra*, *Ammodytes*, *Hypoptychus*, *Trachurus*, *Brachyopsis*, *Pholis*, *Opisthocentrus*, *Zoarces*, *Ascoldia*, *Pholidapus,* and *Etrumeus*. More specifically, AFP has been identified in the following fish species: *Myoxocephalus stelleri* (Tilesius), *Gymnocanthus herzensteini* (Jordan et Starks), *Myoxocephalus polyacanthocephalus* (Pallas), *Hemilepidotus jordani* (Bean), *Furcina osimae* (Jordan et Starks), *Hypomesus pretiosus japonicus* (Brevoort), *Hypomesus olidus* (Pallas), *Spirinchus lanceolatus* (Hikita), *Mallotus villosus* (Muller), *Pleurogrammus monopterygius* (Pallas), *Sebastes steindachneri* (Hilgendorf), *Clupea pallasii* (Valenciennes), *Limanda herzensteini* (Jordan et Snyder), *Limanda punctatissima* (Steindachner), *Limanda schrenki* (Schmidt), *Limanda aspera* (Pallas), *Liopsetta obscura* (Herzenstein), *Clidoderma asperrimum* (Temminck et Schlegel), *Cleisthenes pinetorum herzensteini* (Schmidt), *Microctomus achne* (Jordan et Starks), *Lepidopsetta mochigarei* (Snyder), *Platichthys stellatus* (Pallas), *Kareius bicoloratus* (Basilewsky), *Eopsetta grigorjewi* (Herzenstein), *Gadus macrocephalus* (Tilesius), *Theragra chalcogramma* (Pallas), *Ammodytes personatus* (Girard), *Hypoptychus dybowskii* (Steindachner), *Trachurus japonicus* (Temminck et Schlegel), *Brachyopsis rostratus* (Tilesius), *Pholis picta* (Kner), *Opisthocentrus ocellatus* (Tilesius), *Zoarces elongatus* (Kner), *Ascoldia variegata knipowitschi* (Soldatov), *Pholidapous dybowskii* (Steindachner), and *Etrumeus microps*. The muscle homogenates of these fishes can be used as source material for the mass preparation of AFP (Tsuda and Miura 2002).

17.2.7 AFP Purified from Fish Muscle

The AFPs isolated from fish muscle homogenates include type I AFP from barfin plaice (*Liposetta pinnifasciata*), Ca^{2+}-dependent type II AFP from Japanese smelt (*Hypomesus nipponensis*), Ca^{2+}-independent type II AFP from longsnout poacher (*Brachyopsis rostratus*), type III AFP from notched-fin eelpout (*Zoarces elongatus* Kner), and AFGP from saffron cod (*Eleginus gracilis*). These fish were all captured in the northern sea area (~40° latitude) during the winter season (January to March). These results suggest that fish species capable of synthesizing AFPs in vivo are living not only in polar areas but also in the mid-latitudes of the northern and southern hemispheres. The amino acid sequences of AFPs from these fishes are similar, but not identical, to that of their counterparts in polar fishes. For example, the sequence of an isoform of the barfin plaice-derived AFP is D^{1}TASDAAAAAA ATAAAAAAAAAATAKAAAEAAAATAAA AR^{40}-NH_2, which is a typical type I AFP sequence comprising 3 tandem repeats of the 11-residue consensus sequence TX_{10}, where X is mostly Ala. However, this isoform is more Ala-rich (75%) than the other type I AFP isoforms (67%) and uniquely exhibits ultrahigh water solubility (~650 mg/mL) (Mahatabuddin et al. 2017). This AFP isoform is capable of binding to entire surfaces of an ice crystal, including the basal plane, in a concentration-dependent manner. The Ca^{2+}-

independent type II AFPs from longsnout poacher and sea raven share approximately 67% sequence identity. These two fishes look very different but belong to the same suborder Cottoidei. NfeAFP consists of at least 13 isoforms of type III AFP, which are divided into SP (NfeAFP1–6) and QAE (NfeAFP7–13), and the latter is subdivided into QAE1 (NfeAFP7–10) and QAE2 (NfeAFP11–13). The QAE1 isoforms are fully active as antifreezes, while the QAE2 and SP isoforms have the ability to shape ice crystals into hexagonal trapezohedrons but cannot arrest ice growth. The substitution of three surface-exposed amino acids of the defective QAE2 isoform (NfeAFP11) converts it into a fully active QAE1-like isoform (Garnham et al. 2012). The location of ice-like water in this triple mutant (NfeAFP11-V9Q/V19L/G20V) was suggested by NMR spectroscopy (Kumeta et al. 2013).

Type I–III AFPs have been isolated primarily from the plasma of winter-caught fishes. The purification and characterization of the above type I–III AFPs demonstrates the usefulness of fish muscle homogenates as the starting material for mass preparation of native AFP (Fig. 17.1), which avoids the need to handle and bleed live fish. In the case of type I AFP from barfin plaice, approximately 6 g of the protein was extracted from 2 kg of muscle homogenate; from this extract, approximately 1 g of the native AFP of 95% purity was recovered. In general, various enzymes degrade the proteins in a fish body immediately after the death. Nevertheless, AFGP and type I–III AFPs can be purified from fish bodies sold in general food markets as well as packaged dry foods composed of fish bodies (Nishimiya 2008).

17.3 Mechanical Features of AFPs

17.3.1 Unit Structure of Single Ice Crystal

Under 1 atmosphere of pressure, vast numbers of water molecules are organized to create a single ice crystal, whose unit structure is a hexagon defined by a_1–a_3 and c-axes under 1 atmospheric pressure (Fig. 17.2a) (Hobbs 1974). The regularly spaced water molecules of this unit create eight surfaces denoted as two basal and six first prism planes, where the hexagonal arrangement is only observed in the basal plane. If this hexagonal unit is cut from the top to the bottom along the c-axis so as to include two alternate corners, the created slice (dashed square) is defined as a secondary prism plane. All the other slices of this hexagonal unit are called pyramidal planes. The Miller-Bravais indices have been used to numerically represent different ice planes (Hew and Yang 1992; Olijve et al. 2016a, b). For example, the index (0001) represents two basal planes normal to the c-axis, (10–10) shows the six primary prism planes, and (20–21) shows the six pyramidal planes formed between one edge and a midpoint of the two respective basal planes. AFP is a macromolecule capable of binding to the set of water molecules constructing a specific ice plane (Fig. 17.2b). The AFP species termed "moderately active AFP" targets the water molecules that construct the first prism plane, secondary prism plane, or pyramidal plane, but not the basal plane. In contrast, the species termed "hyperactive AFP" targets all sets of water molecules constructing a single ice crystal, including the basal plane (Scotter et al. 2006).

17.3.2 Ice-Shaping Ability

A single ice crystal consists of water molecules in a hexagonal arrangement, while in solution the crystal forms a round disk-like shape so as to minimize surface energy (Fig.17.2c–*a*). Inherently, the growth rate of this circular ice crystal toward the a-axis is ~100 times faster than that along the c-axis (Hobbs 1974). If the solution contains an AFP species that binds to the water molecules constructing a certain ice plane, the ice crystal growth is only allowed between the bound AFPs, according to the "Gibbs-Thomson effect" (Fig. 17.2c–*b*) (Yeh and Feeney 1996; Anklam and Firoozabadi 2005). The resultant convex ice front sandwiched between the AFPs is energetically unfavorable for further binding of water molecules, terminating its growth to form a flat, AFP-accumulated surface

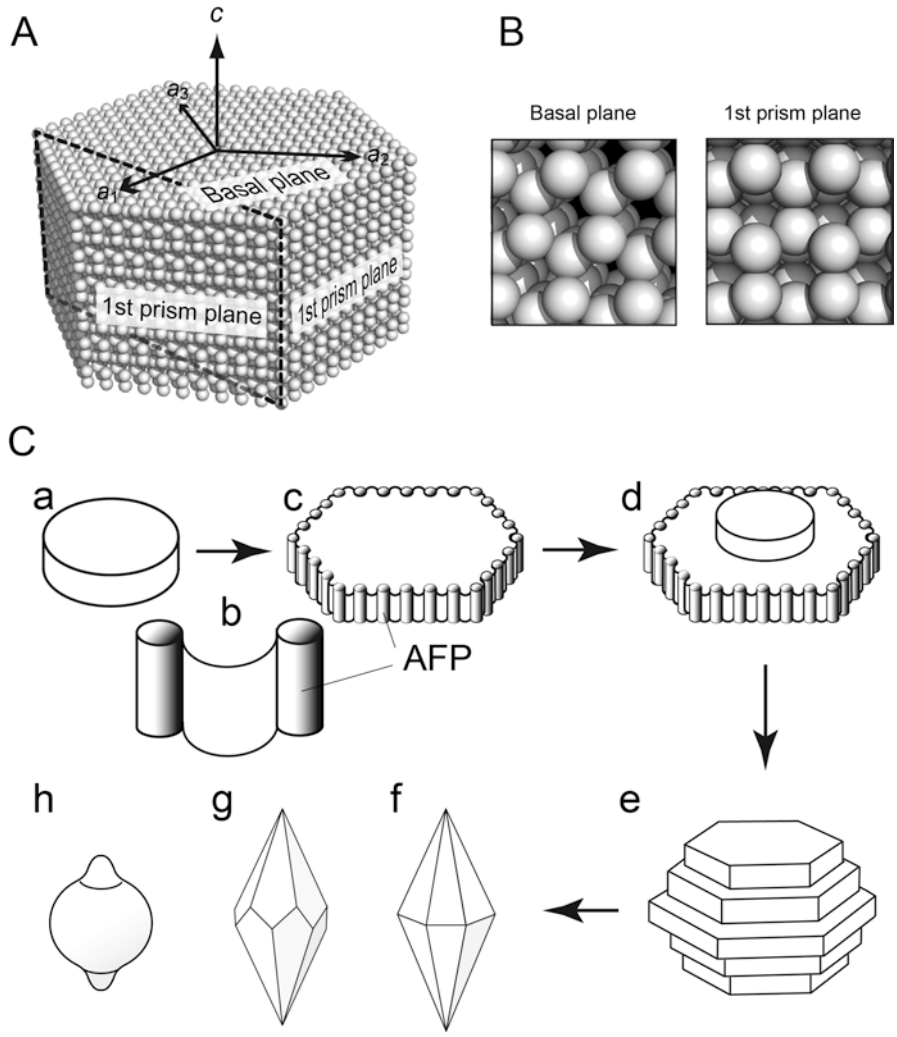


Fig. 17.2 (**a**) A hexagonal ice crystal unit defined with a_1–a_3 and *c*-axes locating two basal and six first prism planes. Dashed square is defined as secondary prism plane. (**b**) Expanded view of oxygen atoms organized in the ice crystal. (**c**) AFP-induced shape change of a single ice crystal. (*a*) A disk-shaped ice crystal, (*b*) convex ice front created between the AFPs represented by cylinders, (*c*) a hexagonal ice plate with AFP-accumulated surface, (*d*) a smaller ice disk generated on the hexagonal ice plate, (*e*) smaller hexagonal ice plates repeatedly stacked on the initial plate, (*f*) a hexagonal bipyramidal ice crystal, (*g*) a hexagonal trapezohedron ice crystal, (*h*) a lemon-shaped ice crystal

(Fig. 17.2c–*c*). This process transfers the disk-shaped ice crystal into a hexagonal plate and allows generation of a new disk on that plate through a mechanism termed "two-dimensional nucleation" (Fig. 17.2c–*d*) (Sazaki et al. 2010). AFPs further accumulate on the 6 planes grown from the new disk as well as the hinge region between the disk and the basal plane, which again modifies the smaller disk into a hexagonal ice plate. Repeated AFP binding and new disk generation cause successive stacking of smaller hexagonal ice plates in the direction of the *c*-axis (Fig. 17.2c–*e*), forming a hexagonal bipyramid onto which millions of AFPs are adsorbed (Fig.17.2c–*f*) (Takamichi et al. 2009). This unique ice crystal morphology is further modified into its derivative forms such as the hexagonal trapezohedron or the lemon-like shape

(Fig. 17.2c–*g*, *h*) (Zhang and Laursen 1999; Bar-Dolev et al. 2012). It should be noted that the ice hexagonal plate remains unchanged and is not modified into a bipyramid at a concentration of 30–50 μg/mL fish-derived AFPs, termed the "critical ice-shaping concentration" (CISC) (Mahatabuddin et al. 2016).

17.3.3 Thermal Hysteresis Activity

For a solution containing no AFP, only a slight change in temperature (e.g., ±0.01 °C) affects the growing and melting of disk-shaped ice crystals in the solution. This observation indicates the equality of T_f and T_m, showing no TH value for this solution. In contrast, the two temperatures are differentiated in an AFP solution. For example, a lemon-shaped ice crystal created in an AFP solution is stably observed regardless of the temperature down to −5 °C. Upon further lowering of the temperature to −5.3 °C, the ice crystal undergoes bursting growth from portions unprotected by the AFP. The bursting-growth temperature is referred to as the nonequilibrium freezing point (T_f). The melting temperature (T_m) of −0.1 °C should be determined separately. For this particular AFP solution, the TH was determined to be 5.2 °C by subtracting T_m from T_f (TH = $T_f - T_m$). Therefore, the TH value represents a certain temperature range in which an AFP-accumulated ice crystal neither grows nor melts (Davies 2014). The TH is commonly measured using a nanoliter osmometer, an instrument in which submicroliter volumes of an AFP solution are introduced in an oil droplet that is temperature-controlled by a Peltier device. The combined use of an ordinary photomicroscope system and a temperature-controlling device, such as a Linkam THMS 600 microscope stage (Linkam Scientific Instruments Ltd., Tadworth, Surrey, UK), has also been used for TH determination (Takamichi et al. 2007). A recently developed sonocrystallization method was used to determine the TH for several AFPs. This method does not use a single ice crystal and obtains much smaller TH values than those reported (Olijve et al. 2016a, b).

17.3.4 Ice-Binding Specificity

To identify target ice plane of an AFP species, a unique method was developed for determining the fluorescence-based ice plane affinity (FIPA) of AFP on a single ice crystal hemisphere (Fig. 17.3) (Garnham et al. 2010; Basu et al. 2014). In this method, a macroscopic single ice crystal is initially prepared in a cylindrical mold. Following determination of its *c*-axis using a polarizer, a half-cut of this cylindrical crystal with a known orientation is mounted on a chilled probe. By submerging it into a hemispherical cup containing degassed water at −0.5°C for 1–2 h, an ice crystal hemisphere with a 2 to 3-cm diameter is created. A method called "ice pitting" (Garnham et al. 2010) generates a six-sided star mark indicating the a_1–a_3 directions on the polar region. The ice crystal hemisphere with known orientation is then mounted onto a chilled probe so as to face down the desired ice plane (Fig. 17.3a). Following 1–2 h incubation with a solution of a fluorescence-labeled AFP, the FIPA pattern can be illuminated on the ice hemisphere under UV light. If six equally distant ellipses are illuminated on the equator as illustrated in Fig. 17.3b–*a*, it shows that AFP binds to six equivalent prism planes. Similarly, the illumination of six ellipses on the mid-latitude (Fig. 17.3b–*b*) implies the AFP binding to six equivalent pyramidal planes. An AFP species that binds to both basal and prism planes provides the FIPA pattern like Fig. 17.3b–*c* (Kondo et al. 2012). A type I AFP capable of binding to whole ice planes gave the pattern like Fig. 17.3b–*d* (Mahatabuddin et al. 2017), on which the six-sided star mark is illustrated.

17.4 AFP Function and Applications

17.4.1 Ice Recrystallization Inhibition

The natural ice block is an assembly of a vast number of single ice crystals of nonuniform size. At subzero temperatures, large ice crystals grow at the expense of smaller ones by a mechanism

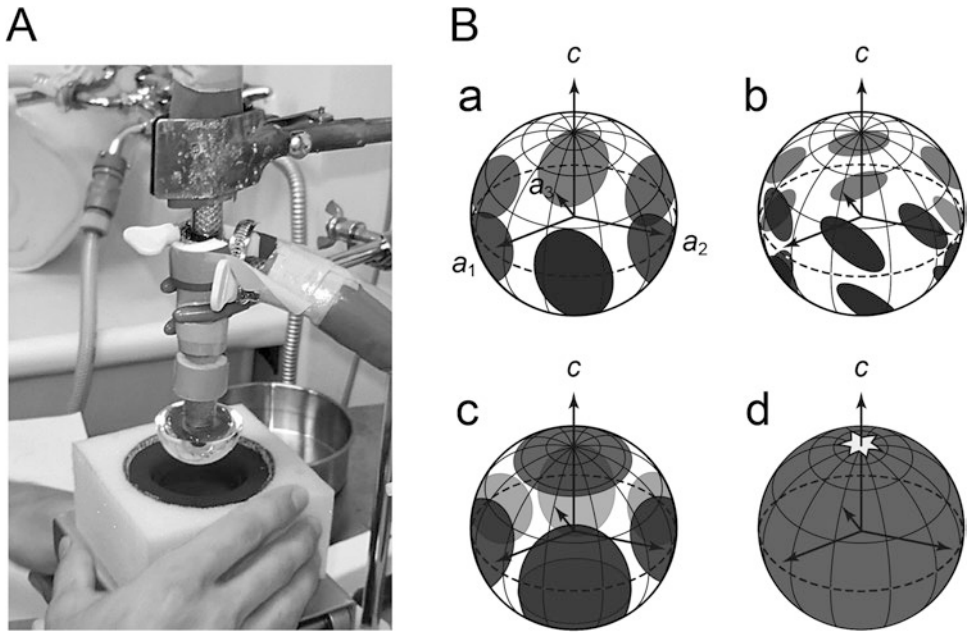


Fig. 17.3 Fluorescence-based ice plane affinity (FIPA) of AFP. (**a**) Experimental setup for observing the FIPA pattern on a single ice crystal hemisphere. The hemisphere mounted on a frosty probe is soaked into 0.01–0.2 mg/ml solution of a fluorescence-labeled AFP to observe its binding onto specific ice planes. (**b**) Typical FIPA patterns illustrated on a globular single ice crystal: (*a*) prism planes observed as the ellipses on the equator (dashed line) (AFPII, AFGP); (*b*) pyramidal planes (AFPI); (*c*) prism, pyramidal, and basal planes (fungal AFP); (*d*) whole ice planes (bacterial and insect AFPs). A six-sided star mark created with ice-pitting method (Garnham et al. 2010) indicates the a_1–a_3 directions of the ice crystal

known as LSW theory or Ostwald ripening (Lifshitz and Slyozov 1961; Kahlweit 1975). The resultant expansion of the ice grains and their uniting process is called ice recrystallization, which is accelerated at higher subzero temperatures. This phenomenon physically destroys the texture of any water-containing materials from inside and outside, as in the freeze-thaw cycle of cryopreservation. AFP and its derivative substances can inhibit ice recrystallization at very low concentrations (Fig. 17.4), with tremendous applicability to frozen foods, cryosurgery, and cryopreservation. Although the mechanism of the AFP-dependent ice recrystallization inhibition (IRI) is unclear, AFPs are thought to bind to the surface of ice grains to prevent their growth and uniting.

Knight et al. (1984) first reported the IRI activity of AFP and then developed the splat-cooling assay (Knight et al. 1988), in which an aliquot of a solution is dropped onto cold metal (−78 °C) to form an ice disk. The disk consists of numerous fine-grained single ice crystals that progress time-dependent recrystallization, which is analyzed by time-lapse photography at −8 °C. This method has been modified for more advanced applications, including the sucrose-sandwich-splat assay (Smallwood et al. 1999) and the glass capillary technique (Tomczak et al. 2003), which are capable of evaluating the AFP concentration representing the IRI endpoint. Domain recognition software and the circle Hough transform (CHT) algorithm have been further developed to extract the average grain size from the time-lapse snapshots, enabling more precise evaluation of ice recrystallization rates (Jackman et al. 2007; Olijve et al. 2016a, b). Significantly, no perfect correlation was detected between high TH and high IRI, although involvement of the same ice-binding residues is indicated in both activities (Yu et al. 2010).

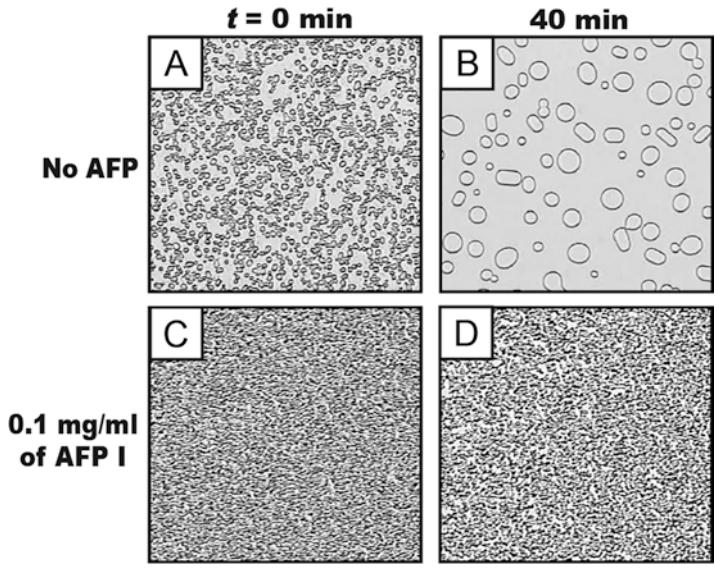


Fig. 17.4 Photomicroscope snapshots of ice crystals containing 40% sucrose in the absence (**a** and **b**) and presence (**c** and **d**) of type I AFP (AFPI). (**a**) The single ice crystals forming dispersion state captured at $t = 0$. (**b**) The ice crystals become larger at $t = 40$ min according to the ice recrystallization process. (**c**) Addition of AFP strongly inhibits growth of ice crystals to keep them small at $t = 0$. (**d**) The ice crystals neither grow nor merge together at $t = 40$ min according to the AFP's ability of recrystallization inhibition

17.4.2 Cell Cryopreservation with AFP

In general, harmful ice formation and recrystallization occur both inside and outside of cells during freezing for storage. To overcome this problem, a two-step freezing method is used in which cells suspended in a preservation fluid are first cooled to near 0 °C. This step freezes only the material outside of the cell, which pulls the water in the cell toward the outside, causing dehydration. In the next step, the cells are frozen entirely with liquid nitrogen (−196 °C). The dehydration lessens ice crystal formation within the cells during this step. This method generally uses a programmable freezer and takes approximately 2–3 h. Another method is vitrification, in which the cells are fast-frozen to −196 °C with highly concentrated (1–3 M) cryoprotectants (e.g., DMSO, acetamide, propylene glycol) that penetrate the cells. In this method, ice crystal formation is prevented both inside and outside of the cells. These protectants are extremely toxic in a liquid state. Hence, the cells and detergents must be frozen quickly after mixing to prevent cell death before freezing. The cells are also damaged during the thawing process by both the toxicity of the detergents and ice recrystallization. The detergents used in the two-step freezing method are also toxic, while they are much more diluted than those used in vitrification (Yarely and Ramos 2010b). Table 17.1 lists the past examples of cell and tissue cryopreservation using AFP. In cold-tolerant organisms, AFPs function in combination with various organic and inorganic substances such as glucose, glycerol, lipids, ions, mineral salts, carbohydrates, and peptides. Thus, AFP performance will be maximized by using optimal combinations of these substances, which differs between applications.

17.4.3 Cell Hypothermic Preservation with AFP

Cryopreservation of cells is not always necessary in the field of livestock farming and regenerative medicine. In such cases, the cells are cooled to +4 °C for hypothermia, stored for 24–48 h, and re-warmed to use for transplanta-

Table 17.1 Examples of cells and tissue cryopreservation using AFP

Cell type	Conditions			References
0Rat kidney	*Tm*AFP	−4°C	61.5 μmol/L	Tomalty et al. (2017)
Mouse ovary	AFPIII	Vitrification	10 mg/mL	Kim et al. (2017)
Bovine oocyte	AFGP8	Vitrification	1 mmol/L	Liang et al. (2016)
Buffalo sperm	AFGP	Two-step	1 μg/mL	Qadeer et al. (2015)
Mouse ovary	AFPIII	Vitrification	20 mg/mL	Lee et al. (2015)
Marine diatom	LeIBP	Two-step	100 μg/mL	Koh et al. (2015)
Buffalo sperm	AFPIII	Two-step	0.1 μg/mL	Qadeer et al. (2014)
Rabbit sperm	AFPIII	Two-step	1 μg/mL	Nishijima et al. (2014)
Rabbit embryo	AFPIII	Two-step	500 ng/mL	Nishijima et al. (2014)
Seabream sperm	AFPI, III	Two-step	1 μg/mL	Beirao et al. (2012)
Mouse oocyte	AFPIII	Vitrification	500 ng/mL	Jo et al. (2012)
Red blood cell	LeIBP	Two-step	800 μg/mL	Lee et al. (2012)
Mouse oocyte	AFPIII	Vitrification	500 ng/mL	Jo et al. (2011)
Seabream embryo	AFPI	−10°C	N/A	Robles et al. (2007)
Rat islet	syAFGP	Two-step	500 μg/mL	Matsumoto et al. (2006)
Bovine sperm	AFPI	Two-step	1 μg/mL	Prathalingam et al. (2006)
Red blood cell	AFPI, II, III	Two-step	30 μmol/L	Chao et al. (1996)
Rat liver	AFGP	−3°C	1 mg/mL	Rubinsky et al. (1994)
Red blood cell	AFPI	Two-step	150 μg/mL	Carpenter and Hansen (1992)
Mouse embryo	AFGP	Vitrification	40 mg/mL	Rubinsky et al. (1992)

The AFPI−III denotes fish-derived type I−III AFP
The *Tm*AFP, FfIBP, LeIBP, and syAFGP are *Tenebrio molitor* (insect)-derived AFP, *Flavobacterium frigoris*-derived ice-binding protein (IBP), *Leucosporidium* (yeast)-derived IBP, and synthetic AFGP, respectively

tion. Such short-term cell preservation is also called "cell pausing" and offers many advantages over cryopreservation, as it requires no freeze-thaw protocol, no liquid nitrogen, and less toxic detergents (Rubinsky 2003; Robinson et al. 2014). If the preservation period is extended to 1–2 weeks with an optimized solution, the stored cells can be transported to most areas of the world. A battery-powered refrigerator is useful for this purpose, as the transport of liquid nitrogen on airplanes is usually not allowed. A typical preservation fluid used for cell pausing is phosphate-buffered saline (PBS) supplemented with 0.4 v/v BSA (bovine serum albumin), 0.34 mM pyruvate, 5.5 mM glucose, and 15 mM kanamycin. Other examples include Euro-Collins (EC) and University of Wisconsin (UW) solutions (Steffen et al. 1990). While initially developed to preserve specific organs, these solutions are now used for virtually every type of cell and tissue. The performance of these solutions is highly dependent on cell type and varies from cell to cell (Robinson et al. 2014).

In addition to ice binding, fish AFPs are capable of protecting mammalian cells and tissues at 4 °C from hypothermic damage (Table 17.2). Rubinsky et al. (1990) initially discovered this second ability of AFP that could maintain the membrane potential in porcine and bovine oocytes, improving their fertilization rate after preservation for 24 h at 4 °C. In general, temperature lowering causes a transition of the lipid bilayer from liquid crystal to gel phase, making it leaky and subject to the losing the balance between intra- and extracellular substances (Hays et al. 1996). In the case of human blood platelets, unwanted physiological activation occurs below 20 °C, the temperature at which the membrane phase transition occurs. Blood banks currently preserve human platelets at 22 °C for a maximum of 4 days to suppress their activation. Fish AFPs are hypothesized to block such cold-induced changes in the lipid bilayer, which is expected to maintain its integrity through hydrophobic interactions (Tomczak et al. 2002). Tablin et al. (1996) examined this AFP ability for use in the chilled preservation of human platelets, reporting that

Table 17.2 Examples of nonfreezing hypothermic preservation of cells and tissues with AFP

Cell type	Conditions			References
Bovine embryo	AFPI, III	10 days	10 mg/mL	Ideta et al. (2015)
Rat insulinoma cell	AFPI, III	5 days	10 mg/mL	Kamijima et al. (2013)
Mouse spermatozoa	AFPIII	5 days	10 mg/mL	Kiga et al. (2011)
Rat neuron	AFPI	8 h	10 mg/mL	Rubinsky et al. (2010)
Human hepatoma cell	AFPIII	72 h	10 mg/mL	Hirano et al. (2008)
Rat heart	AFPI, III	32 h	15 mg/mL	Amir et al. (2004)
Carp spermatozoa	AFGP	5 days	10 mg/mL	Karanova et al. (2002)
Sheep embryo	AFPI, III	4 days	1–10 mg/mL	Baguisi et al. (1997)
Human platelet	AFGP	21 days	10 mg/mL	Tablin et al. (1996)
Rat liver	AFPIII	24 h	10 mg/mL	Lee et al. (1992)
Bovine oocyte	AFPI, II, III	24 h	10 mg/mL	Rubinsky et al. (1991)
Pig oocyte	AFGP	24 h	10 mg/mL	Rubinsky et al. (1990)

These experiments were performed at +4 °C with an exception of rat heart that was supercooled to −1.3 °C with AFP (Amir et al. 2004)

they can be stored at 4 °C for at least 21 days. Amir et al. (2004) further reported a successful subzero nonfreezing preservation of rat hearts using AFPs, in which the hearts were stored at −1.3 °C for 32 h in supercooled UW solution supplemented with 15 mg/mL AFP. The lifetime of human hepatoma cells (Hirano et al. 2008) and rat insulinoma cells (Kamijima et al. 2013) was prolonged to 72–120 h by treatment with AFPs dissolved in EC solution. More recently, bovine embryos were kept alive for 10 days at 4 °C in 10 mg/mL AFP dissolved in culture medium 199 containing 20% (v/v) fetal bovine serum (FBS) and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and gave rise to a healthy calf (Ideta et al. 2015).

17.4.4 Creation of Freeze-Tolerant Gels and High-Porosity Ceramics

Many water-containing materials have a gel structure, in which a polymer network holds water molecules to create an intertangled texture with desired viscoelasticity. Examples include jelly, noodles, dough, boiled eggs, cakes, and tofu, all of which are damaged by the freeze-thaw process due to ice block formation that alters the internal texture (Delgado and Sun 2001). In the case of 0.5% (w/v) agarose gel, storage for hours in a home freezer (−18 °C) destroys the gel structure (Fig. 17.5, left) and its ability to hold water, which flows out of the material during thawing. AFP accumulates on embryonic ice crystals to inhibit their formation of ice blocks, preserving the polymer network and its water-holding property during the freeze-thaw process (Fig. 17.5, right). For 0.5% agarose gel, only 0.05–0.1 mg/mL AFP is required for protection. The use of AFP to maintain gel structures will be applied to many different targets.

Products containing numerous pores, known as "porous materials," include filters, membranes, absorbers, insulators, and catalyst carriers. One of the challenges of nanotechnology is to develop distinctive porous materials with a texture that is extremely fine and homogenously organized. The "gelation freezing method" has been developed to fabricate such porous materials, for which AFP was found to make a significant contribution (Fukushima et al. 2013). The first step of this method is to prepare a solution containing gelatin, ceramic powder, and AFP. This mixture is then cooled to form a gel structure and placed on a freezing plate to induce unidirectional freezing. This step creates numerous tiny ice crystals in the bottom of the gel and elongates them from the bottom to the top. Because AFP binds to the surface of the elongating ice crystals, extremely sharpened and uniformly aligned ice needles are created in

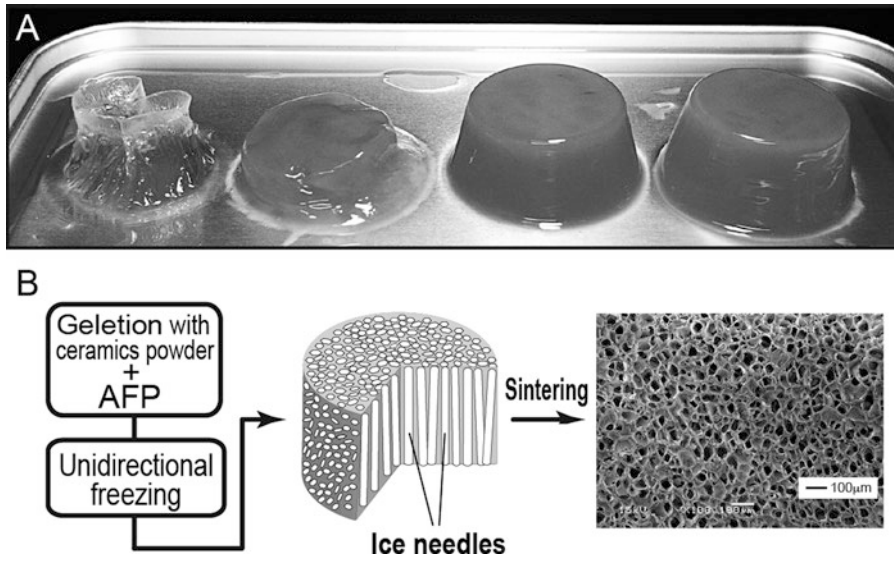


Fig. 17.5 (**a**) Photographs of 0.5% (w/v) agarose gel containing 0, 0.01, 0.05, and 0.1 mg/ml (left → right) of type I AFP after freeze-thawing. (**b**) Fabrication of high-porosity ceramics by sintering a frozen gel that contains ceramics powder and AFP. An optimized amount of AFP creates extremely thin ice needles in the frozen gel, which becomes tiny pores after sintering

the frozen gel. Such ice needles are hardly created nor elongated without AFP. In the last step, the frozen gel composed of numerous ice needles is sintered at 1000–1500 °C. The gelatin and AFP are burned out, while the ceramic powder remains, resulting in a ceramic material containing unidirectionally aligned dendritic pores (Fig. 17.5). The creation of such an ice-driven structure inside a gel or other water-containing material is one of the promising uses of AFP.

17.5 Conclusions and Future Perspectives

Knowledge of AFP application obtained over the last decades demonstrated that there are promising usages of AFP in various industrial and medical fields. Advances in both physicochemical and structural biologic techniques as well as genetic methods have significant contribution and allowed us to gain an unprecedented insight into the mechanism of ice binding and membrane protection of AFP. Maximum applicational studies have been performed in the field of cell preservation, which is presumably attributed to the scarcity of the AFP sample that had to be purified for a long time from blood sera of living polar fishes. For technological applications, biomolecules must be both functionally useful and available in large quantities. Examples include cold-active enzymes used in laundry detergents, pharmaceutical proteins, diagnostic antibodies, and bio-ethanol. This review focused on natural fish AFP, as it is a mixture of AFP isoforms whose activity is greater than any single isoform and is available in larger quantities. Development of prototypes of cell preservation solution, freeze-tolerant gel, and porous material indeed consume purified fish AFP samples in at least grams to achieve statistical significance. Further quantity of AFP will enable development of more large-scale techniques in industrial and medical fields. Approval from government agencies will be required for the medical use of AFP with regard to its toxicity, mutagenicity, and carcinogenicity, for which the sample should be prepared in a facility that satisfies the regulation of good manufacturing practice. Further studies on

numerous aspects of AFP will provide the information necessary to overcome these problems.

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Development and Application of Cryoprotectants

18

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Abstract

Cryopreservation involves the preservation of biological materials, including cells, embryos, tissues, and organs, at ultra-low temperatures (in a state of suspended animation), for a long period of time, and in a way that allows them to be restored whenever required. Freezing of biological samples is generally accompanied by numerous undesirable outcomes such as intra- and extracellular freezing damage and osmotic stress. To prevent these adverse effects, cryoprotective agents (CPAs) are added to biological materials before freezing. Over the years, a number of CPAs have been identified and developed and have been employed successfully for numerous applications. Here, we review the history and development of cryoprotectants and the current understanding of the cryopreservation process. We conclude with a discussion about the application of cryopreservation for various clinical and academic studies.

Keywords

Cryopreservation · Vitrification · Polymers · Cells · Tissues

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Abbreviations

ART	Assisted reproductive technology
COOH-PLL	Carboxylated poly-L-lysine
CPA	Cryoprotective agent
DMSO	Dimethyl sulfoxide
HES	Hydroxyethyl starch
PLL	Poly-L-lysine
PVP	Polyvinylpyrrolidone

18.1 Introduction

Our current understanding of cryopreservation stems from studies performed over the last two centuries. However, freezing or cooling at low temperature was identified thousands of years ago as an efficient way of preservation. People in China identified this technique as early as 1000 BC when they began using ice cellars to preserve foods (Archer 2004). Initially, the use of low-temperature preservation was limited mainly to vegetables. Low-temperature storage of vegetables allowed the retention of their quality after harvest because, at low temperatures, the rate of cellular metabolism is decreased, and fruit ripening and plant senescence are delayed (Muthukumarappan and Tiwari 2010).

The first attempt at cryopreservation of a biological sample was done by Italian physiologist Spallanzani, in 1776, when he froze sperm

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(semen) (Spallanzani 1776). About six decades later, the first patent for cold preservation was issued to H. Benjamin in 1842 for the preservation of food in ice and salt brine (Birdgeye and Fitzgerald 1932). The pioneering study by Polge and his team in 1949 led to the discovery of glycerol as a potent cryoprotectant (Polge et al. 1949). Following this finding, numerous studies were carried out, and researchers discovered a number of similar additives that could preserve cells after freeze-thawing. Another milestone in this field was the discovery that dimethyl sulfoxide (DMSO) could very efficiently preserve red blood cells. Following these studies, numerous cryoprotective agents (CPAs), including sugars, polymers, and proteins, were discovered. These discoveries have revolutionized several areas of biotechnology such as plant and animal breeding. In clinical studies, advancements in the field of cryopreservation have enabled the preservation of eggs, embryos, organs, and tissue-engineered constructs.

18.2 Development of CPAs

Investigation of freezing, winter hardiness, and frost resistance by biologists in the nineteenth and early part of the twentieth century gave rise to some of the earliest known concepts of cryopreservation. Initial attempts at cryopreservation involved freezing biological samples at low temperatures without any additives. However, it was soon realized that freezing cells and other biological materials resulted in extensive freeze-induced damage such as intra- and extracellular ice crystal formation and osmotic stress. Therefore, researchers started adding chemicals to cells, which they termed "cryoprotectants." Cryoprotectant is a broad term that can be used to describe any external additive which, when added to cells prior to freezing, protects them from various freeze-induced damages and yields high post-thaw cell viability.

18.2.1 Glycerol

The first highly effective CPA was discovered almost accidently. It is believed that glycerol, as a CPA, was discovered by the team of Polge, Smith, and Parkes in 1949 when a mistake in labeling of a bottle in the refrigerator led a researcher to freeze fowl semen in a mixture of glycerol, albumen, and water (Polge et al. 1949). Previously, they used a levulose solution that was not very effective. However, they observed that this new mixture had remarkably high efficiency. After extensive investigations, the actual composition of this unknown mixture was determined, and it was found that glycerol was the active ingredient most responsible for imparting a cryoprotective property to this mixture. One year later, Smith showed that glucose-saline solutions containing 10% and 15% (final concentrations) glycerol prevented hemolysis of red blood cells after freeze-thawing, and the blood could be stored for 7 days at +2 °C without appreciable hemolysis (Smith 1950). This was a significant discovery because, prior to this, no method was available that could prevent, on a large scale, the hemolysis of red blood cells that occurs during freezing and thawing.

A few years later, James Lovelock, a colleague of Polge, Smith, and Parkes at the National Institute for Medical Research in London, UK, reported that damage to cells on freezing occurred due to an increased concentration of electrolytes within the cell and that the presence of glycerol greatly reduced the electrolyte concentration, thus imparting a protective effect (Lovelock 1953a, b). Additionally, Lovelock (1954) found that a number of other neutral solutes, such as glyceryl monoacetate, methanol, and acetamide, showed cryoprotective properties similar to glycerol. He also noted that only nontoxic compounds with a low molecular weight, ability to permeate living cells, and high solubility in aqueous electrolyte solutions had cryoprotective properties. He observed that, although many other solutes had protective properties, the number of solutes

fulfilling these conditions was very low, and glycerol was the only solute known that approached being an ideal protective agent.

18.2.2 DMSO

Although glycerol is a very effective cryoprotectant, a large amount is required for satisfactory results. In addition, some cells, including bovine red blood cells, was found to be impermeable to glycerol. Hence, researchers realized that glycerol cannot be used as a universal solute for preserving all cell types. To overcome this challenge, Lovelock and Bishop began exploring other low molecular weight compounds. They discovered that DMSO had a greater permeability to living cells than glycerol. They observed that DMSO permeated cells (both bovine and human red blood cells) more rapidly than glycerol, and a far lower concentration was required for complete protection against freezing damage (Lovelock and Bishop 1959). At this time, DMSO gained considerable attention globally and was mentioned as a miracle compound. Following this, DMSO was found to be an effective agent for the cryopreservation of homograft valves, and its use was implemented in clinical medicine (Kuleshova and Hutmacher 2008). Additional studies have since been carried out successfully using DMSO as a cryoprotectant, e.g., preservation of pig leukocytes at liquid nitrogen temperature using a medium containing 10% DMSO and 50% pig serum (Koch et al. 1991) and preservation of third-stage larvae of *Angiostrongylus cantonensis* (Mahajan and Renapurkar 1993).

18.2.3 Polymers

DMSO, glycerol, and other low molecular weight CPAs mentioned in the preceding sections are membrane-permeable compounds. As such, their removal from cells has presented a substantial obstacle for their clinical application. Many studies have reported methods of removal after thawing. Moreover, DMSO is toxic to lymphocytes and causes osmotic lysis of cells. Hence, researchers explored the possibility of using polymers for cryopreservation. Polymeric CPAs, because of their high molecular weight, cannot permeate cell membranes like DMSO, glycerol, and other low molecular weight CPAs. This gives them the advantage of being easily removed after thawing by a simple washing step.

18.2.3.1 Polyvinylpyrrolidone (PVP)

PVP was identified at Linde Laboratories as an additive exhibiting an unusual ability to protect cells from freezing injury (reviewed by Meryman) (Meryman 1964). Richards and Persidsky reported the preservation of bone marrow using PVP and found that PVP with a molecular weight of 30 kDa in a 10% solution gave results superior to 15% glycerol in the preservation of bone marrow (Richards and Persidsky 1961). In a subsequent study, they postulated that PVP imparted protection by coating the cell, thus plugging pores in the cell membrane. This prevented changes in the intracellular electrolyte concentration, ice formation, and excessive dehydration (Persidsky and Richards 1962). They also pointed out that PVP possessed the added advantage of permitting freeze-drying, which is not possible with preservation using glycerol. Since then, many studies reported using PVP for cryopreservation of mammalian cells (Ashwood-Smith et al. 1972) and freezing of human lymphocytes (Damjanovic and Thomas 1974). Subsequently, most studies relied on PVP for clinical applications of cryopreservation.

18.2.3.2 Hydroxyethyl Starch (HES)

The development of a polymeric CPA and its success in clinical applications led researchers to explore other polymeric compounds as cryopreservatives. Prior to the discovery of HES as a CPA, it was used to separate granulocytes from human blood in the sedimentation of buffy coats to concentrate leukocytes. Similar to PVP, HES

is also widely used as a plasma expander (Stolzing et al. 2012). This prompted studies of HES as a potential CPA. Garzon et al. (1967) and Knorpp et al. (1967) independently proposed HES for the cryopreservation of erythrocytes. Knorpp observed an average recovery of erythrocytes in vitro in the range of 97–99%. He argued that HES, being a starch, offered the advantage over PVP of being metabolized. Hence, it is not retained in the body thereby eliminating the need for extensive processing of blood after thawing prior to transfusion. To date, HES has been used successfully for cryopreserving a number of biological materials including pancreatic islets (Kenmochi et al. 2008), granulocytes (Lionetti et al. 1975), hamster cells (Ashwood-Smith et al. 1972), and some other cell types (Stolzing et al. 2012).

Mechanistic investigations showed HES cryopreserved cells by absorbing water molecules from outside the membrane and keeping them thermally inert in a glassy state, without experiencing any phase transition during cooling (Körber and Scheiwe 1980). This reduced the viscosity, triggering an increase in the rate of dehydration (avoiding intracellular ice crystal formation as well as chilling injury), kinetically inhibiting ice formation, and decreasing the cooling rate required for optimal survival during vitrification (Connor and Ashwood-Smith 1973; Takahashi et al. 1988).

18.2.3.3 Polyampholytes

Polyampholytes have both positive and negative charges on the polymer chain. Matsumura and Hyon (2009) discovered that polyampholytes had excellent cryoprotective properties. They prepared the polyampholyte, carboxylated ε-poly-L-lysine (COOH-PLL), by introducing succinic anhydride to neutralize positively charged poly-L-lysine moieties. COOH-PLL showed excellent compatibility with various cell types, even in the absence of any animal-derived proteins like fetal bovine serum. They also showed that COOH-PLL exhibited significantly less cytotoxicity than DMSO (Fig. 18.1).

In their subsequent studies, Matsumura et al. (2010) showed that COOH-PLL could be used to cryopreserve numerous cell types including primary, derived, adhesive, and floating cells and to enable long-term preservation (up to 24 months) (Matsumura et al. 2013). To further explore the efficiency of polyampholytes as cryopreservatives, they synthesized completely synthetic compounds via reversible addition fragmentation chain transfer polymerization (Rajan et al. 2013). These synthetic polyampholytes also showed excellent cell viability and good biocompatibility. An interesting finding of this study was that the introduction of a small amount of hydrophobicity (butyl or octyl methacrylate) to the polyampholytes markedly increased their cryoprotective property. Preliminary mechanistic investigations revealed that polyampholytes cryopreserved cells by strongly interacting with cell membranes, which enabled them to protect the membrane from various freeze-induced damages, including inhibition of ice recrystallization. Addition of hydrophobicity led to stronger interactions with the cell membrane (Rajan et al. 2016).

To further investigate the characteristics and mechanism of the antifreeze effect of COOH-PLL, Vorontsov et al. (2014) studied its effect on the crystallization of ice. They found that adsorption of large biological molecules during ice crystallization had a non-steady-state character and occurred at a slower rate than the embedding of crystal growth units.

18.2.4 Sugars

Sugars were perhaps the first (protective) additives used prior to freezing (Maximow 1912), and both mono- and disaccharides are used frequently as CPAs. Even when the activity of glycerol as a cryopreservative was discovered accidently due to the wrong labeling of the bottle in the refrigerator, the intended solution was levulose (a ketonic monosaccharide) (Polge et al. 1949). Addition of sugars helps regulate the osmotic pressure of diluents. This is done by inducing cell dehydration and avoiding ice crystal formation (Leibo and Songsasen 2002).

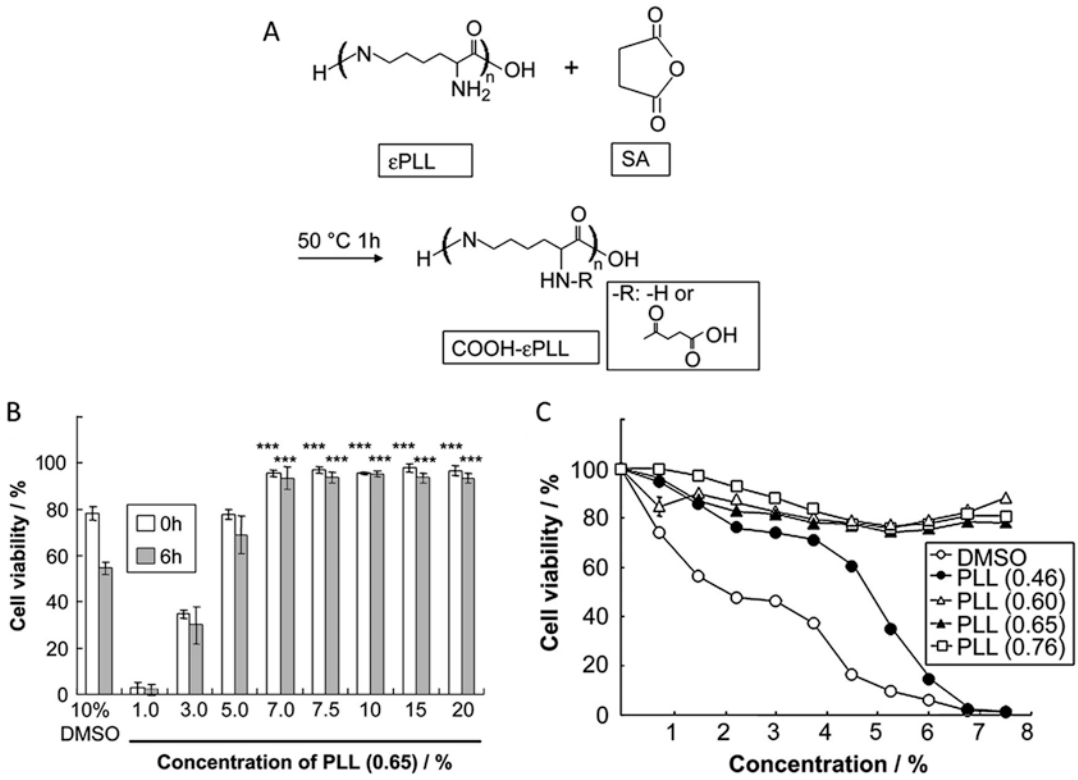

Fig. 18.1 (**a**) Synthesis of carboxylated *ε*-poly-L-lysine (COOH-εPLL). (**b**) L929 cells were cryopreserved with various concentrations of PLL (0.65). Cell viability immediately (white bars) and 6 h (gray bars) after thawing at 37 °C. Data are expressed as means ± SD for three independent experiments (five samples each). ***$P < 0.001$ vs. 10% DMSO for the corresponding time (0 or 6 h). (**c**) Cytotoxicity of DMSO (open circles) and PLL (0.46) (closed circles), PLL (0.60) (open triangles), PLL (0.65) (closed triangles), and PLL (0.76) (open squares). L929 cells were incubated with the indicated concentration of each compound for 48 h, followed by the MTT assay. Data are presented as mean percentages of untreated cells ± SD for three independent experiments (eight samples each). (Reprinted with permission from: Matsumura and Hyon 2009)

Although both mono- and disaccharides have been used for the low-temperature preservation of cell lines, disaccharides such as sucrose and trehalose are more widely used compared to monosaccharides. Independent studies on mice by Wang et al. (2014) and on bovine spermatogonial stem cells by Kim et al. (2015) showed that sucrose and trehalose are the most effective cryoprotectants among all disaccharides. Hincha et al. (2006) reported that sugars stabilize membranes by interacting with the polar head groups of phospholipids. Furthermore, disaccharides, especially trehalose, enhance glass formation and reduce the amount of CPA required for vitrification (Fuller 2004). Trehalose efficiently cryopreserves mammalian cells (Eroglu et al. 2000), hematopoietic progenitor cells (Buchanan et al. 2004), human pancreatic islets (Beattie et al. 1997), and primary human hepatocytes (Katenz et al. 2007). On the other hand, sucrose is effective in cryopreserving human plasma low-density lipoproteins (Rumsey et al. 1992), human sperm (Hossain and Osuamkpe 2007), and pig spermatogonial stem cells (Pan et al. 2017).

18.3 Cooling Rates

The rate of freezing is one crucial factor controlling the efficiency of cryopreservation. Mazur (1977) noted that the relationship between cell survival and cooling rate followed an inverted

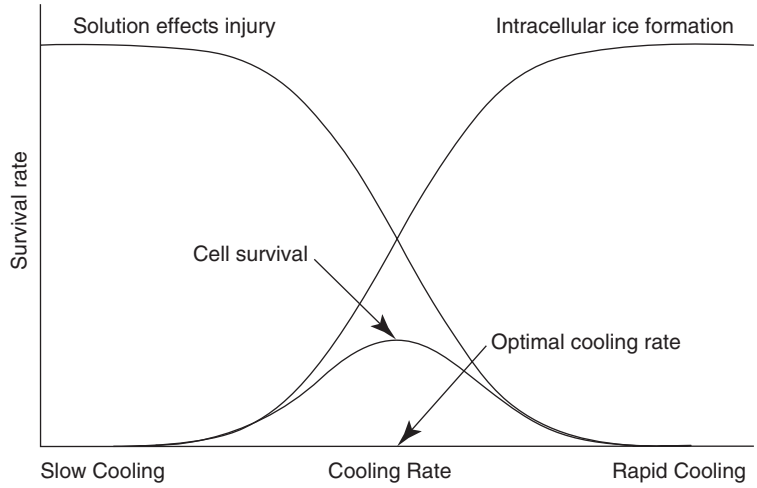

Fig. 18.2 Plot of the interaction between cooling rate, "solution effect" injury, ice formation injury, and cell survival. At low cooling rates, "solution effects" are the dominant factor in cell damage, but as cooling rates increase and exposure time decreases, these effects are minimized. Conversely, at high cooling rates, intracellular ice formation is the dominant factor in cell damage, and as cooling rates are decreased, the likelihood of intracellular ice formation decreases. The combination of these two effects implies that there will be an inverted "U"-shaped survival curve and an optimal cooling rate that minimizes both the solution effects and intracellular ice formation. (Reprinted with permission from Benson (2012), Theriogenology, 78, 1682–1699)

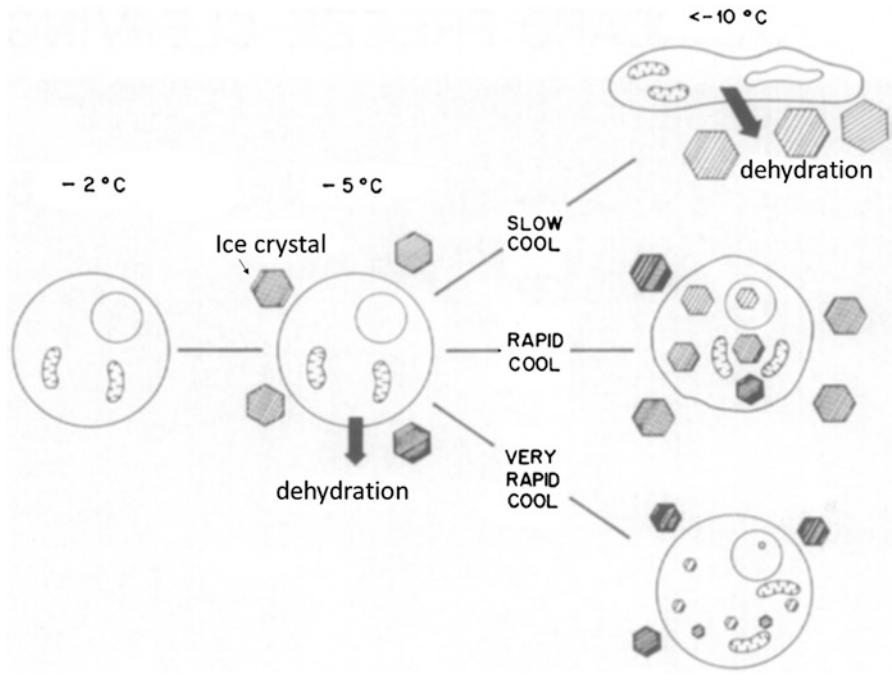

Fig. 18.3 Schematic of the physical events in cells during freezing. (Reprinted with permission from Mazur 1977)

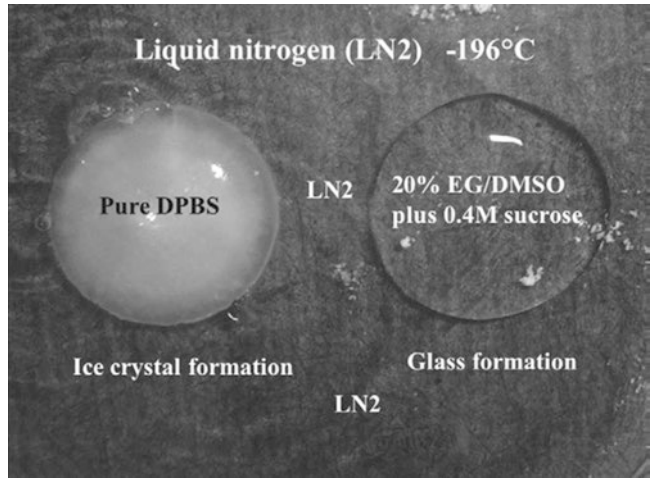

Fig. 18.4 Image of vitrification. Two droplets of different solutions were plunged directly into liquid nitrogen. The left droplet is pure Dulbecco's phosphate-buffered saline (DPBS) and shows ice crystallization. In contrast, the right droplet contains an equimolar combination of 20% ethylene glycol (EG) and dimethyl sulfoxide (DMSO) with 0.4 M sucrose in DPBS. This droplet solidified without ice crystallization (glassy, vitrified state). (Reprinted with permission from Liebermann et al. (2003), Reprod Biomed Online 7:623–633)

U-shaped curve. Each system (cells or other biological materials) had an optimum cooling rate that depended on various factors (Fig. 18.2) (Benson et al. 2012). Very slow cooling led to excessive cell dehydration. In contrast, cooling at very high rates led to rapid water flow through the membrane, which resulted in an inconsistent pressure distribution across the membrane, thus causing sudden changes in the size and shape of the membrane.

18.3.1 Slow Cooling

The primary cause of cell death during freezing is intracellular ice formation. Slow cooling allows dehydration, reducing the amount of water inside cells that could potentially form ice. This occurs because, during slow cooling, extracellular ice forms first, resulting in a differential water gradient across the cell membrane which ultimately moves intracellular water to the outside, thus avoiding intracellular ice formation (Fig. 18.3). At the same time, excessive dehydration increases the intracellular concentration of solutes (solution effect) which can lead to osmotic shock. The amount and rate at which intracellular water is lost depends on the permeability of the specific cell type. More permeable cells show far greater tolerance toward fast cooling than less permeable cells (Mazur et al. 1972).

18.3.2 Vitrification

Vitrification (derived from the Latin word *vitreum,* which means glass) refers to the process whereby a substance in transformed from a liquid into a glassy state. This is achieved by cooling at a very fast rate (around 15,000–30,000 °C/min), which leads to the formation of amorphous ice. Because of rapid cooling, water molecules have insufficient time to form ice crystals, hence transforming directly to a glassy state (Fig. 18.4). During vitrification, an extreme elevation in viscosity of the intracellular fluid is achieved. This causes molecules to become immobilized, i.e., no longer remain a liquid. Mechanical damage caused by ice crystals is averted which prevents major damage to cells. The vitrification process involves exposing the sample to high concentrations of a CPA for a short period at ambient temperature, followed by cooling at a very fast rate in liquid nitrogen. Although vitrification was used

previously in other applications, Luyet (1937) is believed to have been the first to use this technique for preservation of biological systems. He noted that crystallization was incompatible with living systems and should be avoided whenever possible, and thus explored the possibility of using vitrification. He attempted to vitrify living matter without the addition of any CPA and identified the possibility of attaining structurally immobile states by rapid cooling. The first successful cryopreservation of animal cells by vitrification was done by Rall and Fahy (1985). Since then, vitrification has been widely used for the preservation of numerous clinically important materials like cells, tissues, embryos, and oocytes (Matsumura et al. 2015).

18.4 Applications of Cryopreservation

Over the years, cryopreservation has become an indispensable tool in numerous biological, medical, aquatic, and agricultural fields and in the clinical practice of reproductive technology. Cryopreservation not only reduces cost and labor by eliminating the need to regularly culture cell lines that are no longer required but also is extremely important in the event of microbial contaminations, natural disasters, or alterations of genetic expression in later generations. Moreover, cryopreservation of reproductive cells and embryos not only helps couples who cannot reproduce naturally but also eliminates the risk of contamination and disease. These goals have been achieved by new regulations that make it mandatory to quarantine donated cells and the need to perform requisite tests for HIV (Hunter 1995). Moreover, it is also very important to preserve strains that are not currently required but could find important applications in the future. The technique of cryopreservation has been employed in various clinical studies, cryosurgery, ecology, plant physiology, and food preservation. In the following sections, we will briefly elaborate on the development and application of cryopreservation in different fields.

18.4.1 Cryopreservation of Embryos

Findlay et al. (2007) defined a human embryo as "a discrete entity that has arisen from either: the first mitotic division when fertilization of a human oocyte by a human sperm is complete or any other process that initiates organized development of a biological entity with a human nuclear genome or altered human nuclear genome that has the potential to develop up to, or beyond, the stage at which the primitive streak appears, and has not yet reached 8 weeks of development since the first mitotic division." Embryo cryopreservation can enable the preservation of endangered species and allow the storage of genetic information. At the same time, embryo cryopreservation is mainly used to preserve unused embryos after in vitro fertilization. These embryos may be used at a later stage if the patient fails to conceive or for donation. Polge (1977) reported the advantages of cryopreserving embryos in a book section.

Many attempts have been made to cryopreserve embryos. DMSO, glycerol, dextran, and sucrose have been tried, but without appreciable success (Whittingham and Wales 1969; Whittingham 1971). Whittingham et al. (1972) reported the cryopreservation of mouse embryos using 7.5% PVP at −79 °C and a cooling rate of 1 °C/s for 30 min. He found that most of the eight-cell and early blastocysts could be recovered after freeze-thawing. However, this method could not be used for storing embryos for more than 30 min, and two-cell embryos could not be recovered.

The first successful cryopreservation of (mouse) embryos was reported by Whittingham, Leibo, and Mazur in 1972 (Whittingham et al. 1972). They used slow cooling (0.3–2 °C/min) and slow warming (4–25 °C/min) and observed that 50–70% of the frozen and thawed early embryos developed into blastocysts in culture. Of the surviving embryos, 65% resulted in pregnancy. This method soon became popular because of its efficiency and reproducibility. Since then, many studies have been reported for the cryopreservation of rat (Whittingham 1975), rabbit (Whittingham and Adams 1976), sheep

(Willadsen et al. 1976), and cow embryos (Wilmut and Rowson 1973), all employing the slow cooling technique. Rall and Fahy (1985) reported vitrification of embryos using a 25% vitrification solution (named VS1) composed of 20.5% DMSO, 15.5% acetamide, 10% propylene glycol, and 6% polyethylene glycol. A high proportion of embryos survived cryopreservation using this technique. Although this technique had its own shortcomings, it showed the potential of vitrification for preserving embryos.

The first successful pregnancy after cryopreserving human embryos was reported by Trounson and Mohr (1983). Those embryos were frozen and stored as eight-cell embryos for 4 months in liquid nitrogen. Since then, many successes and pregnancies have been reported using freeze-thawed embryos (Hredzak et al. 2005; Rama Raju et al. 2005; Li et al. 2007).

18.4.2 Cryopreservation of Gametes

Reproductive cells of an organism are called gametes or sex cells. Gametes produced by the male are called spermatozoa (sperm cells) and those by the female are called oocytes (ova or eggs). They are haploid cells that fuse with each other during fertilization and form a new diploid organism. Cryopreservation of human gametes is a very important tool and has become an integral part of assisted reproductive technology (ART) procedures.

18.4.2.1 Cryopreservation of Oocytes

Immature egg cells, such as female gametocytes or germ cells, which are involved in the reproductive process, are known as oocytes. They are produced in the ovary via female gametogenesis, mature during the menstrual cycle, and further develop into fertilizable eggs. A female is born with a finite number of oocytes. The majority of these are lost through a process known as ovarian follicle atresia (Hsueh et al. 1994; Welt 2008). The remaining eggs are released during each menstrual cycle, thereby controlling a woman's capacity to reproduce and making the reproductive life span of a woman finite.

Women undergoing chemotherapy and radiotherapy for cancer and other illnesses are at a greater risk of follicular atresia, thus causing primary ovarian insufficiency (ACOG 2014). There can be several other reasons for this disorder such fragile X premutation and mosaicism for monosomy X (both are genetic conditions). However, the cause of about 90% of the cases of primary ovarian insufficiency remains a mystery (Nelson 2009). This makes the preservation of oocytes extremely crucial, and women with these risk factors needing ART may be prime candidates for oocyte cryopreservation. In addition, preservation of oocytes can be equally useful for women who wish to defer childbearing until later in life for reasons such as facing difficulty in finding a suitable partner or who believe child bearing at an early stage of their career may be detrimental.

Although cryopreservation of embryos is an established and successful ART technique, it requires that the women have either a male partner or use a sperm donor, neither of which may always be desirable. In addition, women with the risk factors mentioned above, and who have difficulty in finding a suitable partner, may consider banking mature oocytes as a reasonable fertility-preserving alternative.

Compared to semen cryopreservation, oocyte cryopreservation is very difficult as they are more prone to intra- and extracellular ice crystallization, which is fatal to cells. Moreover, oocytes are very sensitive, and slow cooling could result in chilling injury (Vincent and Johnson 1992; Bernard and Fuller 1996; Leibo et al. 1996). Hence, oocytes are frozen using vitrification, a difficult and inefficient procedure. Numerous research centers worldwide have reported successful pregnancies after cryopreservation of oocytes. However, the clinical efficiency remains very low at 2–4%, probably due to poor survival, fertilization problems, and improper development of the cryopreserved oocytes (Matsumura et al. 2015).

One of the earliest successful attempts at oocyte cryopreservation was done by Kuleshova et al. (1999). They reported that a healthy baby girl was born at 37 weeks of gestation after vitri-

fication of mature oocytes from four in vitro fertilization patients. They vitrified 17 oocytes in ethylene glycol and sucrose solution. Eleven of these oocytes survived after vitrification. Since then, numerous other studies have been reported where relatively good success has been achieved (Cobo et al. 2008; Rienzi et al. 2010; Cobo et al. 2010; Parmegiani et al. 2011).

Many modifications have been done to improve the efficiency of vitrification such as using:

- Different CPAs like DMSO (Chen 1986), ethylene glycol, ethylene glycol and sucrose solution (Kuleshova et al. 1999), and COOH-PLL (Watanabe et al. 2013)
- Different methods like open pulled straw vitrification (Vajta et al. 1998), ultrarapid cooling, vitrification in straws (Watanabe et al. 2013), nanoliter droplet vitrification (Zhang et al. 2012), and the Cryotop method (Cobo et al. 2008)
- Different temperatures and different rates of cooling (Konc et al. 2014)

Although it is still not 100% efficient and safe, vitrification of oocytes is quickly becoming a viable option for women who want to defer childbearing and is also recommended for patients facing infertility due to chemotherapy or other medical conditions.

18.4.2.2 Cryopreservation of Spermatozoa

Compared to oocytes, spermatozoa are easier to cryopreserve because they are less sensitive to freezing damage due to their high membrane fluidity and low water content. Since the first successful attempts to freeze sperm by Polge et al. (1949), many researchers have attempted to cryopreserve sperm, and there have been many improvements in the technology.

Cryopreservation is used extensively as part of ART to preserve male gametes and provide the opportunity for future fertilization by storing donor and partner spermatozoa. Similar to oocyte preservation, sperm preservation is important during cytotoxic chemotherapy and radiation therapy. Several surgical treatments may also lead to testicular failure or ejaculatory dysfunction. Cryopreservation is also extremely useful for homosexual couples and men with azoospermia (a condition where semen contains no sperm) which may be due to percutaneous epididymal sperm aspiration or testicular sperm extraction (Donnelly et al. 2001). Sperm cryopreservation and banking facilitates the availability of sperm at the time required for insemination. Finally, cryopreserved sperm minimizes the risk of disease transmission to the female partner. This is achieved by storing and using only those samples that are not infected with a disease. Another important reason to cryopreserve sperm cells is their very short life span because, for fertilization, sperm and oocytes must be available at the same time (Miyaoka and Esteves 2013). This may be extremely inconvenient or impossible in some cases. Hence, the availability of banked sperm in such scenarios can be extremely beneficial.

After the discovery of glycerol as a CPA, major improvements were made in sperm cryopreservation techniques. The first pregnancy resulting from cryopreserved human sperm was reported by using glycerol (Bunge and Sherman 1953). For human sperm cryopreservation, both egg yolk and glycerol are commonly added at room temperature. Cryopreservation processing of sperm involves equilibration of the samples with the CPA for several minutes, followed by loading sperm cells into cryovials or straws and subsequent freezing. Behrman and Sawada (2017) proposed a technique where sperm were cooled over a period of 2–4 h in two or three steps, either manually or automatically using a semi-programmable freezer (Behrman and Sawada 2017). It was later observed that slow freezing caused extensive damage to the sperm cells due to ice crystallization (Di Santo et al. 2012). Subsequently, a rapid freezing technique was developed by Sherman. This involved bringing sperm-loaded straws and nitrogen vapors into direct contact for 8–10 min, followed by immersion in liquid nitrogen (Sherman 1990). He also pointed out that glycerol may cause cellular alterations such as the presence of an undulating membrane, changes in the acrosomal internal

membrane, nucleus inhomogeneity, and disorganization in mitochondrial crests. Other nonpermeating CPAs such as DMSO and 1,2-propanediol have also been used for the cryopreservation of sperm cells.

Vitrification has also been employed for sperm cryopreservation. Nawroth et al. (2002) reported the vitrification of human spermatozoa without the addition of any CPAs by direct plunging into liquid nitrogen slush. However, many problems have been reported with this technique, including the risk of infectious agent transmission, because of the direct contact of samples with liquid nitrogen and difficulty in properly labeling the samples. Although slow-freezing remains the preferred procedure for spermatozoa cryopreservation, research is being conducted to develop improved strategies for vitrification.

18.4.3 Cryopreservation of Tissue-Engineered Constructs

Compared to cell suspensions, cryopreservation of regenerated tissues, such as cell sheets and cell constructs, is difficult. Present-day cryopreservation techniques work well for cells but are not equally effective for large cell-biomaterial constructs because membranous structures of the cultured sheets are damaged during the freezing and thawing processes. There is a great demand for the cryopreservation of cell-containing constructs (such as cell sheets and scaffolds) for tissue engineering applications such as producing off-the-shelf tissue-engineered products for clinical needs.

Cryopreservation (slow freezing) of chondrocytes was reported earlier. However, this process resulted in the nucleation and growth of ice crystals within the chondrons. Hence, Pegg and his coworkers identified the need to avoid the crystallization of ice and to establish new vitrification protocols. These researchers showed that chondrocytes isolated from cartilage tissues can be cryopreserved easily using the "liquidus-tracking" method; details may be found in Pegg et al. (2006), which completely eliminates the crystallization of ice and does not require rapid warming. Song et al. (2004) reported the vitrification of rabbit cartilage with more than 80% cell viability. Kuleshova et al. (2007) reviewed the prospect of vitrification for cryopreservation of tissue-engineered constructs.

Jain et al. (2014) prepared a cryoprotective polyampholyte hydrogel in which cells were encapsulated for cryopreservation. Copper-free click chemistry was used to prepare dextran- and COOH-PLL-based hydrogels. Most of the cells frozen in these hydrogels survived after thawing, thus making it a promising system for long-term storage and a suitable off-the-shelf tissue-engineered product. In a recent study, Matsumura et al. reported a new "slow vitrification method" for the cryopreservation of two-dimensional cell constructs using a vitrification solution containing 6.5 M ethylene glycol, 0.5 M sucrose, and 10% COOH-PLL. Vitrification rates of 4.9 and 10.8 °C/min resulted in remarkable viability of cultured human mesenchymal stem cell monolayers after freezing, and less apoptosis was induced (Matsumura et al. 2016).

18.4.4 Vitrification of Tissue-Engineered Intestine

Tissue-engineered intestine is being studied for the treatment of short bowel syndrome, which results from the loss of intestinal tissue and is a major cause of intestinal failure. Tissue-engineered intestines have the potential to eliminate the need for intestine transplantation. Choi and Vacanti showed that tissue-engineered small intestines can be produced by in vivo implantation of organoid units (OU) (Choi and Vacanti 1997). This was a major discovery in the field of tissue-engineered intestines. OU are multicellular clusters of epithelial and mesenchymal progenitor cells harvested from native intestine (Spurrier and Grikscheit 2013). Spurrier et al. (2014) showed that tissue-engineered intestines could be generated using preserved (vitrified) murine and human donor cells. Viability in the range of 89–93% was observed with murine and human OU. Tissue-engineered small intestine

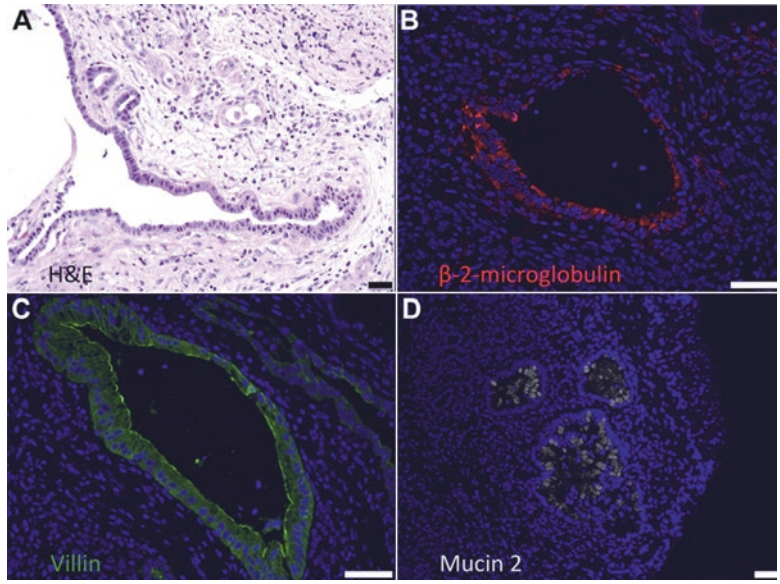

Fig. 18.5 Human tissue-engineered small intestine generated from vitrified organoid units. (**a**) Hematoxylin and eosin (H&E) staining showing a rudimentary epithelial layer. (**b**) Immunofluorescence staining for β-2-microglobulin confirming the human origin of a tissue-engineered small intestine construct. Immunofluorescence staining for villin (**c**) and mucin 2 (**d**) showing the presence of differentiated enterocytes and goblet cells, respectively. Nuclei are stained blue with 4′,6-diamidino-2-phenylindole. Scale bars, 50 μm. (Reprinted with permission from Spurrier et al. 2014)

was then successfully generated from the cryopreserved OU (Fig. 18.5).

Other tissue-engineered materials, such as bone (Yin et al. 2009) and blood vessels (Pegg et al. 1997; Elder et al. 2005), have also been preserved using vitrification techniques and different CPAs. Over the years, cryopreservation techniques have been extended to other systems including bacteria, yeasts, fungi, amoeba, algae, plant protoplasts, seeds, and thylakoid membranes (Day and McLellan 1995).

18.5 Conclusions and Future Trends

Since the early attempts of low temperature preservation of biological materials using snow or refrigeration, the field of cryopreservation has grown tremendously. The major milestone in this field was the discovery of CPAs. These have made possible the long-term cryopreservation of almost all kinds of cells, tissues, and organs. Both cell-permeating (DMSO and glycerol) and non-permeating (polymeric) CPAs have been identified, and their mode of action has been studied. Identification of effective vitrification techniques has facilitated the cryopreservation of complex materials like tissues and tissue-engineered constructs, bones, intestines, and blood vessels and has helped in the commercialization and industrialization of tissue engineering. Additionally, development of cryopreservation techniques for gametes and embryos has enabled the creation of sperm and embryo banks that have helped numerous couples to conceive and enabled the birth of endangered species of animals.

Although a number of technological advances have been made in the last 200 years, especially in the last 70 years, cryopreservation remains an under-investigated topic compared to other topics in the field of tissue engineering. Even though cryopreservation of embryos and human gametes has been done for a number of years, and many successful pregnancies have been reported, the success rate is still not sufficient to make it a completely reliable system. Similarly, current vitrification techniques for the preservation of

many systems still involve the use of high concentrations of CPAs, which could be potentially toxic during the process of thawing. Hence, there is a great need to develop newer CPAs and technologies to cryopreserve a wide range of biological materials and allow them to be restored with all their functions. A number of studies are currently in process to develop improved CPAs and cooling methods. In fact, some research groups are trying to cryopreserve human bodies with an aim to restore them to full health at a time when a reliable cure or new technology is available for their illness or disorder.

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Cryopreservation of Plant Genetic Resources

19

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Abstract

Cryopreservation encompasses several interconnect disciplines including physiology and cryophysics. This chapter reviews the current techniques for cryopreservation of plant genetic resources (PGRs). Vitrification is an effective ice crystal avoidance mechanism for hydrated cells and tissues. With any cryopreservation method, whole or partial parts of specimens which are sufficiently dehydrated can be vitrified by rapid cooling in liquid nitrogen (LN). Techniques discussed are the vitrification protocol, encapsulation-vitrification protocol, droplet vitrification protocol (DV), vitrification protocol using cryo-plates (V cryo-plate), and air dehydration protocol using cryo-plates (D cryo-plate). In these DV, V, and D cryo-plate protocols, specimens to be cryopreserved are immersed directly into LN on aluminum foil strips or cryo-plates; removal from LN to rewarming solution results in a high level of plant regrowth with ultrarapid cooling and warming. The protocols were applied to a wide array of plant species including wild and multi-ploid species, although fine tuning of the protocols was required for successful application to specific plant species and lines. These three protocols efficiently complement each other and appear highly promising to facilitate large-scale cryobanking of PGRs in genebanks. Cryo-scanning electron microscopy makes it possible to examine the cellular and water behavior in plant tissues when immersed in LN. It has been verified that tissues cryopreserved by the process of vitrification and the cryo-plate protocols are cryopreservation methods for reliable long-term preservation of PGRs.

Keywords

Cryopreservation · Vitrification · D cryo-plate · V cryo-plate · Intracellular ice formation · Plant genetic resources

Abbreviations

Cryo-plate	Aluminum cryo-plate
Cryo-SEM	Cryo-scanning electron microscopy

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D cryo-plate	Air dehydration protocol using cryo-plates
DV	Droplet vitrification protocol
EV	Encapsulation-vitrification protocol
LN	Liquid nitrogen
LS	Loading solution
MS medium	Murashige and Skoog medium
PGRs	Plant genetic resources
PVS2	Plant vitrification solution 2
V cryo-plate	Vitrification protocol using cryo-plates

19.1 Introduction

Cryopreservation is a method for the long-term storage of plant genetic resources (PGRs) and experimental collections such as algae, cell cultures, and organized plant tissues (Reed 2008). The status of the main cryo-stored germplasm in the world was shown with different materials such as orthodox seeds, dormant buds, and in vitro shoot tips (Niino and Valle-Arizaga 2015). Prior to 1990, the conventional slow-freezing protocol and cryopreservation technique using dormant buds were usually used. New techniques including vitrification, encapsulation dehydration, pregrowth desiccation, and the encapsulation-vitrification protocols have subsequently been developed (Engelmann 2014). With these protocols, explants are usually cooled or warmed in capped cryotubes, with or without plant vitrification solutions. The cooling and warming rates are about 100–200 °C/min and about 80–140 °C/min, respectively (Niino et al. 2013). More recently novel cryopreservation techniques have been developed including the droplet vitrification (DV) protocol, vitrification protocol using aluminum cryo-plates (V cryo-plate), and air dehydration protocol using cryo-plates (D cryo-plate). With these new protocols, specimens to be cryopreserved are immersed directly into liquid nitrogen (LN) or rewarming solution on aluminum foil strips or aluminum cryo-plates (cryo-plates). Their cooling and warming rates are about 4000–5000 °C/min and about 3000–4500 °C/min, respectively, resulting in high regrowth rates after rewarming (Niino et al. 2013).

In this chapter, the cryopreservation techniques discussed are vitrification protocol, encapsulation-vitrification protocol (EV), DV, V cryo-plate, and D cryo-plate protocols. In addition, the cellular and water behavior in tissues during different cryopreservation steps, as shown by cryo-scanning electron microscopy, are presented. The technical advantages of these novel cryopreservation protocols will facilitate cryobanking of PGRs.

19.2 Concept of Vitrification for Cryopreservation

Cryopreservation of shoot tips has been employed in a wide range of plant materials for long-term preservation. To avoid damage by extracellular and intracellular freezing in cells, vitrification has been proposed as a protocol for the cryopreservation of biological materials (Luyet 1937). Sakai (1956) reported that the survival of poplar and salix twigs collected in the winter can survive after LN or liquid helium exposure. Fahy et al. (1984) proposed that vitrification was requisite for survival of biological cells at the temperature of LN. Vitrification can be defined as the transition of water directly from the liquid phase into an amorphous phase or glass while avoiding the formation of crystalline ice. Vitrification is a method for ice crystal-free formation in cells allowing safe cryogenic storage. However, water is very difficult to vitrify because the growth rate of crystals is very fast, even just below freezing point. So, highly concentrated cryopreservation solutions including glycerol and/or ethylene glycol are necessary to vitrify shoot tips. These very viscous cryopreservation solutions are easily supercooled below −70 °C. Vitrification is one way of safe long-term storage of biological materials because it facilitates the cessation of chemical reactions that are required for molecular diffusion in LN, leading to cell dormancy and stability over time (Burke 1986). Thus, vitrification is an effective

ice crystal avoidance mechanism for hydrated cells and tissues.

With any cryopreservation method, whole or partial parts of specimens which are sufficiently dehydrated can be vitrified by rapid cooling in LN. A very useful dehydration solution called plant vitrification solution 2 (PVS2), which contains 30% (w/v) glycerol, 15% (w/v) ethylene glycol, and 15% (w/v) DMSO in culture medium containing 0.4 M sucrose at pH 5.8, was developed by Sakai et al. (1991). The PVS2 is an extremely concentrated solution at about 8 M and easily supercools below −70 °C upon rapid cooling and solidifies into a metastable glass at about −115 °C. Upon warming, the vitrified PVS2 displays a glass transition at about −115 °C, an exothermic devitrification (crystallization) at about −75 °C, and an endothermic melting at about −36 °C (Sakai et al. 1990). These mechanisms require rapid cooling and warming rates for avoiding ice crystal formation in cells and tissues.

19.3 Cryopreservation Techniques of In Vitro Shoot Tips

The in vitro shoot tips are excellent materials for cryopreservation and also have the characteristics of micropropagation potential and genetic stability. The meristematic zone of apical meristems is composed of a relatively homogeneous population of small actively dividing cells with small vacuoles and high nucleocytoplasmic ratio which make them withstand dehydration better than highly vacuolated and differentiated cells.

19.3.1 Vitrification Protocol

With the vitrification protocol, shoot tips and cells must be sufficiently dehydrated with vitrification solution to avoid lethal injury from cooling and warming process. Since Sakai et al. (1991) reported successful cryopreservation of citrus embryonic cells by vitrification with PVS2, many plant species (using mainly shoot tips) have been cryopreserved with this technique. In the vitrification protocol, cells and tissues must be sufficiently dehydrated with PVS2 without injury, vitrifying them by rapid cooling into LN or devitrifying by warming in a warming solution.

High survival of in vitro grown shoot tips after cryopreservation is determined not only by the cryogenic protocol itself but also by the physiological conditions of the materials to be cryopreserved (Niino and Valle-Arizaga 2015). This means that some steps for acquisition of dehydration tolerance or low temperature tolerance are crucial in the cryopreservation procedures. In any vitrification protocol, procedures such as preconditioning, preculture, osmoprotection (with loading solution, LS), PVS2 exposure time, and post-LN handling are vital. The successive steps for the vitrification procedure of gentian (*Gentiana scabra* Bunge) are as follows (Tanaka et al. 2004): Cold-hardened, precultured in vitro shoot tips are placed in 2.0 ml cryotubes and osmoprotected with LS (0.4 M sucrose +2.0 M glycerol) for 30 min. Then the shoot tips are dehydrated PVS2 for 30–40 min at 25 °C (Fig. 19.1). After dehydration, they are suspended in fresh PVS2 in cryotubes, and cryotubes are then plunged into LN. For regeneration, cryotubes are rapidly warmed in a 37 °C water bath for 1–2 min. After warming, PVS2 is replaced with 1.0 M sucrose solution. After incubation for

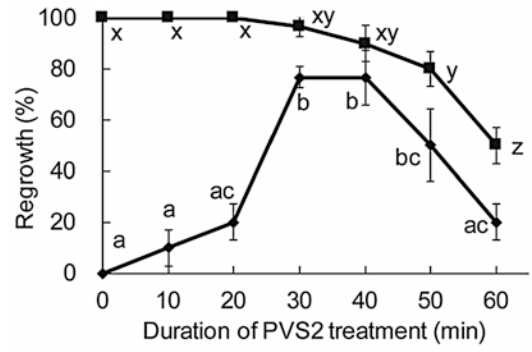

Fig. 19.1 Effect of duration of PVS2 treatment on survival of shoot apices of *Gentian scabra* cooled to −196 °C by the vitrification protocol. Preculture, 0.3 M sucrose at 5 °C for 1 day; osmoprotection, 25 °C for 20 min (Tanaka et al. 2004)

20 min at room temperature, the shoot tips are transferred onto the gentian medium. By this method, the average regrowth of cryopreserved shoot tips of ten gentian cultivars was 49.0%, ranging from 16.7% to 76.7% (Tanaka et al. 2004). As successful results by PVS2 vitrification, more than 200 species and varieties could be successfully stored in LN, such as *Allium sativum*, *Asparagus* spp., *Cymbidium* spp., *Dianthus caryophyllus*, *Malus* spp., *Pyrus* spp., *Trifolium repens*, *Wasabia japonica*, and so on (Sakai et al. 2008). The PVS2 vitrification protocol would allow the widespread cryostorage of not only shoot tips but also cultured cells, somatic embryos, embryonic axes, etc. However, in vitrification procedures, small-size shoot tips are suspended in various solutions employed, which have to be removed and added by repeated pipetting. This often results in damage and loss of shoot tips during course of the cryopreservation protocol. Moreover, vitrification procedures require a precise control of duration of treatment with vitrification solutions due to the narrow range of optimal treatment durations.

19.3.2 Encapsulation-Vitrification Protocol

Different plant species require the successive steps of the vitrification protocol to be optimized for explants survival. In addition, large variations in survival are observed among different lines or cultivars of a given species. Thus, the wide application of cryopreservation requires protocols that can be easily used for a wide range of plant species. The encapsulation-vitrification protocol (EV) was developed to this end. The successive steps of the EV of gentian are as follows (Tanaka et al. 2004). The cold-hardened and precultured in vitro shoot tips are encapsulated with alginate gels and osmoprotected by LS (0.4 M sucrose and 2.0 M glycerol) for 90 min at 25 °C. Then the beads are dehydrated with PVS2 for 120 min at 25 °C (Fig. 19.2) and plunged into LN. For regeneration, cryotubes are rapidly warmed in a 37 °C water bath for 1 min. After warming, PVS2 is replaced with 1.0 M sucrose solution. After incubation for 20 min at room temperature, the shoot tips are transferred onto the gentian medium. By this protocol, the average regrowth of cryopreserved shoot tips of ten gentian cultivars was 73.7%, ranging from 43.3% to 93.3%.

The EV presents the following advantages compared to vitrification protocol: (1) high and uniform survival percentages after cryopreservation, (2) wider spectrum of an optimal duration of the PVS2 treatment, and (3) vigorous and fast regrowth of cryopreserved shoot tips. EV has proved to be as effective as or more effective than the vitrification protocol for cryopreservation of shoot tips of, for example, gentian (*Gentian scabra* Bunge; Tanaka et al. 2004), lily (*Lilium japonicum*; Matsumoto et al. 1996), and wasabi

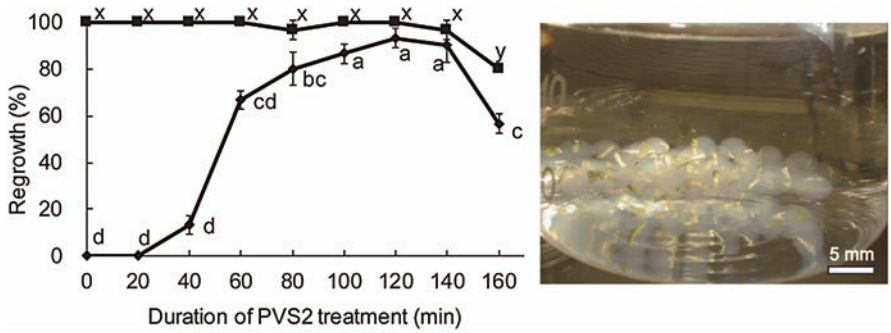

Fig. 19.2 Effect of duration of PVS2 treatment at 25 °C on regrowth of shoot tips of *Gentian scabra* cooled to −196 °C by the encapsulation-vitrification protocol (Left, Tanaka et al. 2004) and alginate beads containing Gentiana shoot tips (Right, Photo Tanaka)

(*Wasabia japonica*; Matsumoto et al. 1995). The reason for high and vigorous regrowth after cryopreservation, when employed with this protocol, was that encapsulation of shoot tips in alginate gel beads decreased the dehydration rate of the shoot tips by PVS2 and direct contact to toxic chemical components of PVS2 (such as DMSO) to shoot tips is avoided. EV might ensure successful cryopreservation of shoot tips that are sensitive to both or either osmotic dehydration and/or exposure to toxic chemicals.

19.3.3 Droplet Vitrification Protocol

The standard vitrification protocol takes place in a cryotube, while the droplet vitrification protocol (DV) is done on aluminum foil strips, and EV adds an encapsulation step to the standard protocol (Panis 2008). Initially the DV was developed for banana cryopreservation and resulted in an increase in the regrowth rate by 40–50% compared to vitrification protocol (Panis et al. 2005). In the DV, shoot tips are dehydrated with a highly concentrated vitrification solution such as PVS2 and then plated on aluminum foil for direct immersion into LN. DV has been notably used for routine cryopreservation of *Musa* spp. (Panis et al. 2005), Korean garlic (Kim et al. 2007), and chrysanthemum (Kim et al. 2011) germplasm collections and gives high recovery. The successive steps of the DV procedure for black chokeberry (*Aronia melanocarpa* Elliot) are as follows (Tanaka et al. 2011). The cold-hardened and precultured in vitro shoot tips are encapsulated with alginate gels and osmoprotected by LS (0.4 M sucrose and 2 M glycerol) for 30 min at 25 °C. Subsequently the shoot tips are dehydrated by 1.0 ml PVS2 at 25 °C for 35 min. The shoot tips are transferred to a drop of PVS2 (about 15 µl) on a strip (5 × 20 mm) of heat-sterilized aluminum foil just 1–2 min prior to plunging into LN or slush nitrogen (SN, −210 °C) with forceps. For regrowth, cryotubes are uncapped, and the strip of aluminum foil with vitrified shoot tips is immersed into 1.0 M sucrose solution in Murashige-Skoog (MS) basal medium at room temperature. After incubation for 30 min, the shoot tips are transferred onto the black chokeberry medium. The optimal duration of exposure to PVS2 at 25 °C in DV was from 15 to 55 min, and 70–80% survival rates (regrowth) could be obtained. With only 5 min exposure to PVS2 at 25 °C, about 50% survival rate was obtained (Fig. 19.3). While in vitrification protocol, the optimal duration of exposure to PVS2 at 25 °C was from 35 to 55 min, and 70–80% survival rates (regrowth) could be obtained. The spectrum of optimal exposure time of DV was broad compared to the vitrification protocol. Surviving shoot tips resumed growth 2 days after rewarming and directly developed shoots without intermediary callus formation (Fig. 19.3).

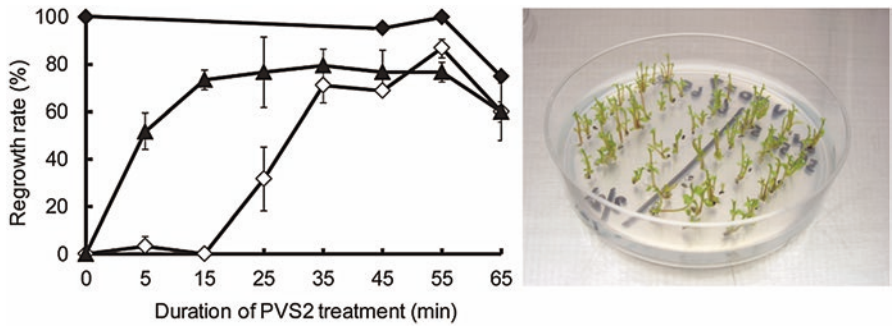

Fig. 19.3 Effect of exposure time to PVS2 at 25 °C on the survival rates of vitrified shoot tips of black chokeberry (Left, Tanaka et al. 2011 modified) and post-thaw regrowth of in vitro shoot tips at 20 days after plating (Right, Photo Tanaka). ◆—◆ Treated control without cooling in LN (-LN). ◇—◇ Vitrification protocol. ▲—▲ Droplet vitrification protocol (DV)

It is well known that the propensity of water to be vitrified depends on both solute concentration and the cooling rate of the system. Both parameters would need to be satisfied for samples to vitrify during cooling and devitrify during warming process. Cooling rates of vitrification and DV protocols were −120 °C/min and −2520 °C/min, and warming rates of them were 120 °C/min and 5340 °C/min, respectively (Tanaka et al. 2011). For DV protocol, the shoot tips on the aluminum foil with 15 µl PVS2 are cooled by direct immersion into LN, while vitrification protocol, shoot tips are immersed in LN on cryotubes containing 0.5 ml PVS2. These faster cooling and warming rates of DV led to higher regrowth rates post-LN storage, resulting to wider spectrum of optimal exposure time (Fig. 19.3).

19.3.4 V Cryo-plate Protocol

In the conventional vitrification procedures including DV, discussed above, small-size shoot tips are suspended in the various solutions employed, which should be removed and added by repeated pipetting. This often results in damage and loss of shoot tips during the cryopreservation protocol. Moreover, vitrification procedures require a precise control of duration of treatment with vitrification solutions, due to the narrow range of optimal treatment durations (Yamamoto et al. 2011). In DV, dehydrated shoot tips need to be transferred to aluminum strips just before immersion in LN, and foil strips with the shoot tips should be transferred in cryotubes. Simpler and more user-friendly cryopreservation procedures are required to facilitate large-scale cryobanking.

Vitrification protocol using aluminum cryo-plates (V cryo-plate) has been developed (Yamamoto et al. 2011). Aluminum cryo-plates were devised to establish a simple, reproducible, and reliable vitrification protocol. The size of aluminum cryo-plate is 7 × 37 × 0.5 mm with ten wells, and cryo-plates can have different well sizes to accommodate explants of different sizes (Fig. 19.4, Niino and Yamamoto 2017). The cryo-plate is designed to fit in 2 ml cryotube. The successive steps of the V cryo-plate of strawberry (*Fragaria×ananassa* Duch.) are as follows (Yamamoto et al. 2011).

Aluminum cryo-plates with ten circular wells (No. 2, Niino and Yamamoto 2017) are placed in a Petri dish. The cold-hardened and precultured in vitro shoot tips are placed in the well filled with sodium alginate solution, one by one, with the tip of a scalpel blade. A calcium solution drop (about 0.3 ml in total) is poured on a section of the aluminum plate and left for 15 min to achieve complete polymerization, resulting the shoot tips to adhere to the cryo-plate through the alginate gels. The cryo-plate with shoot tips is placed in 25 ml pipetting reservoir filled with about 20 ml LS (0.8 M sucrose and 2.0 M glycerol) for 30 min at 25 °C. Then, the cryo-plate is placed in 25 ml pipetting reservoir filled with about 20 ml PVS2 and dehydrated with PVS2 for 50 min at 25 °C (Table 19.1). After dehydration, the cryo-plate is transferred in an uncapped 2 ml plastic cryotube, which is held on a cryo-cane, and directly plunged into LN. For regeneration, the cryo-plate is retrieved from LN and immersed in 2 ml of 1.0 M sucrose solution in a 2 ml cryotube. The shoot tips are incubated in this solution for 15 min at room temperature and then transferred onto the strawberry MS medium (Fig. 19.4). Using this procedure, the average regrowth level of vitrified shoot tips of 15 strawberry cultivars was 81%, ranging from 70% to 97% (Yamamoto et al. 2012a, c).

This V cryo-plate has been adopted for Dalmatian chrysanthemum (Yamamoto et al. 2011), mat rush (Niino et al. 2013), potato

Table 19.1 Effect of different exposure time to PVS2 and preculture days on regrowth of cryopreserved shoot tips of strawberry (*Fragaria* × *ananassa* Duch. cv. "Benifuji") using the aluminum cryo-plate. Different small letters indicate significant differences ($P < 0.05$). (Yamamoto et al. 2012a, c modified)

Exposure time by PVS2	Regrowth (% ± SE)	
	Preculture 1 day	Preculture 2 days
30 min	$37^{a} \pm 6$	$50^{a} \pm 10$
40 min	$47^{a} \pm 6$	$63^{ab} \pm 6$
50 min	$67^{a} \pm 6$	$80^{b} \pm 10$
60 min	$43^{a} \pm 6$	$53^{a} \pm 6$

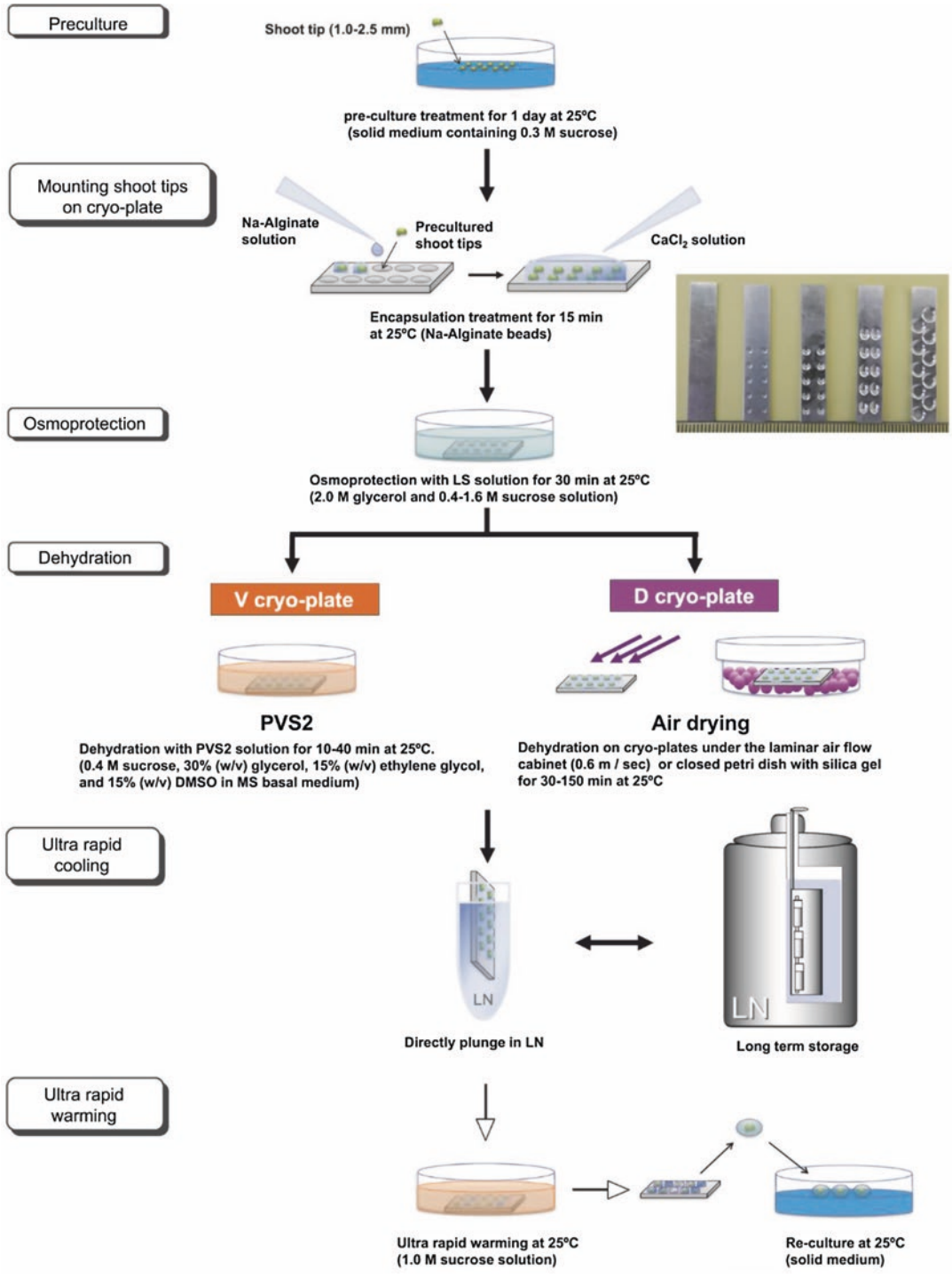

Fig. 19.4 Illustration of V and D cryo-plate protocols

(Yamamoto et al. 2015), sugarcane (Rafique et al. 2015), *Chenopodium odorum* (Engelmann-Sylvestre and Engelmann 2015), mint (Yamamoto et al. 2012a, c), mulberry (Yamamoto et al. 2012a, c), carnation (Sekizawa et al. 2011), cherry plum and plum (Vujović et al. 2015), Cleomaceae (da Silva Cordeiro et al. 2015), and *Perilla* (Matsumoto et al. 2014) shoot tips/buds with high regrowth after cryopreservation. The V cryo-plate has the following advantages: (1) most handling can perform on the cryo-plate with shoot tips, (2) possibility of injuring and losing shoot tips is low, (3) easy and efficient treatment with LS and PVS, (4) rapid cooling and warming, (5) high regrowth percentages, and (6) training staff is not difficult.

19.3.5 D Cryo-plate Protocol

Damage to specimens cryopreserved using the V cryo-plate can occur at different stages. They include chemical toxicity of PVS2, physical damage to apical meristems during excision, and osmotic stress due to the PVS2 treatment being too long. Avoiding the use of PVS2, excising only young leaves and sheaths of shoot tips or buds, and using uniform and healthy shoot tips with high water content may limit damage (Niino et al. 2013). The D cryo-plate was developed to address these concerns (Niino et al. 2013). The successive steps of the D cryo-plate procedure for mat rush (*Juncus decipiens* Nakai) are as follows (Niino et al. 2013, 2014).

Aluminum cryo-plates with ten elliptical wells (No. 3, Niino and Yamamoto 2017) are placed in a Petri dish. The cold-hardened and precultured in vitro buds are placed in the well filled with sodium alginate solution with 0.4 M sucrose, one by one, with the tip of a scalpel blade. Then, the sodium alginate solution (about 2 µl) is poured again on the buds using a micropipette. A calcium solution drop with 0.4 M sucrose (about 0.3 ml in total) is poured on a section of the aluminum plate and left for 15 min to achieve complete polymerization, resulting the buds to adhere to the cryo-plate through the alginate gels. The cryo-plate with buds is placed in 25 ml pipetting reservoir filled with about 20 ml LS (1.0 M sucrose and 2.0 M glycerol) for 30 min at 25 °C. Then, the buds on the cryo-plates are dehydrated by placing the cryo-plates for 150 min either in the air current of a laminar flow cabinet or in Petri dishes containing silica gel at 25 °C, with 40–50% RH (Table 19.2). After dehydration, the cryo-plate is transferred to an uncapped 2 ml plastic cryotube, which is held on a cryocane, and directly plunged into LN. For regeneration, the cryo-plate is retrieved from LN and immersed in 2 ml of 1.0 M sucrose solution in a 2 ml cryotube. The shoot tips are incubated in this solution for 15 min at room temperature and then transferred onto the mat rush MS medium (Fig. 19.4). Using this procedure, the average regrowth level of vitrified buds of 20 mat rush lines was 88%, ranging from 70.0% to 96.7% (Niino et al. 2013).

Table 19.2 Effect of dehydration protocol and of dehydration duration on moisture content and on regrowth (%) of cryopreserved buds of mat rush line (*Juncus decipiens* Nakai "Hiroshima 4gou (1)" using the D cryo-plate procedure. (Niino et al. 2014 modified)

Duration of dehydration	Laminar airflow		Silica gel	
	Buds MC (% FW)	Regrowth (% ± SE)	Buds MC (% FW)	Regrowth (% ± SE)
90 min	31	76.7 ± 3.3[a]	30	80.0 ± 5.8[a]
120 min	28	90.0 ± 0[a]	27	86.7 ± 3.3[a]
150 min	26	93.3 ± 3.3[a]	26	90.0 ± 0[a]
180 min	25	73.3 ± 3.3[a]	25	70.0 ± 5.8[a]

The D cryo-plate has been adopted for potato (Yamamoto et al. 2015; Valle Arizaga et al. 2017), sugarcane (Rafique et al. 2016), *Chenopodium odorum* (Engelmann-Sylvestre and Engelmann 2015), persimmon (Matsumoto et al. 2015), cherry plum and plum (Vujović et al. 2015), date palm (Salma et al. 2014), and blueberry (Dhungana et al. 2017) shoot tips/buds/polyembryonic masses with high regrowth after cryopreservation. The D cryo-plate protocol presented overcomes problems associated with sensitivity to PVS2, insufficient or excessive dehydration, damage to and loss of material during excision and manipulations, besides the advantages of the V cryo-plate (Niino et al. 2013).

19.4 Behavior of Cells and Water in Cells of Plant Shoot Tips Stored in LN

To achieve effective vitrification with plant shoot tips, samples are generally treated with a vitrification solution consisting of several cryoprotectants such as DMSO, glycerol, ethylene glycol, and sugars. For successful cryopreservation, the materials must be vitrified before immersion in LN without lethal ice crystal formation in cells. When vitrification occurs successfully, different plant species can be preserved in LN for a long period (Niino 2000; Matsumoto et al. 2013). In addition, cryopreserved shoot tips show high viability, without phenotypic changes on regrowth, and showing no genetic changes in succeeding generations (Hirai and Sakai 2001; Maki et al. 2015; Matsumoto et al. 2013). If optimized cryopreservation protocols are used, there should be no or little damage to cells after long-term storage. However, there are few reports related to cell ultrastructure and water behavior in cells when stored in LN. Cryo-scanning electron microscopy (Cryo-SEM) has been employed to observe ultrastructure of cells in shoot tips stored in LN.

19.4.1 Cryo-scanning Electron Microscopy of Shoot Tips Cooled with Liquid Propane

The ultrastructure of cells in shoot tips of gentian plants was observed with Cryo-SEM after fixation by rapid cooling. Gentian shoot tips at each step of the vitrification procedure were immersed in liquid propane, transferred into LN, and attached to a specimen holder under LN conditions. The holder with a shoot tip was then immediately transferred and fixed on the cold stage of the pre-evacuation chamber at a vacuum of 1×10^{-4} torr, equilibrated to −105 °C of a Cryo-SEM (JSM840-a, JEOL Ltd, Tokyo, Japan). The shoot tip was then fractured with a cold knife under a binocular microscope, etched slightly for 2 min and then the fractured surface covered with evaporated platinum and carbon. The holder was transferred to the cold stage of the SEM, kept at −160 °C, and images were photographed by secondary emission mode with an accelerating voltage of 5 kV (Fujikawa et al. 1988).

The ultrastructure of cells in shoot was observed with Cryo-SEM after fixation by rapid cooling. The shoot tips with preculture on MS medium containing 0.3 M sucrose at 5 °C for 1 day followed by 20 days of cold hardening resulted in formation of ice crystals evenly distributed not only in vacuoles but also in the cytoplasm and nucleus after rapid cooling in liquid propane (Fig. 19.5a). There were no plasmolyzed cells observed in any parts of the samples. In contrast, with samples treated with LS after preculture and then cooled, no ice crystals were apparent in cells, but many cells were plasmolyzed with contracted vacuoles (Fig. 19.5b). After successive treatments with the LS and PVS2, cells were the same as those treated with the LS. No ice crystals were observed in cells, but cells were plasmolyzed with small contracted vacuoles (Fig. 19.5c). Thus, no apparent differences were detected by Cryo-SEM in samples treated only with the LS compared to those treated with LS plus PVS2.

These results show that there is no apparent ice crystal formation in cells of gentian shoot tips when properly treated with PVS2 and subsequently cooled with cryogens. In addition, after PVS2 treatment, many cells of shoot tips exhibited considerable plasmolysis and formation of small vesicles within cells. In contrast, shoot tips without PVS2 treatment contained many large ice crystals throughout cells after cooling with LN and had no survival after warming. Further research is required using transmission electron microscopy technique to obtain more detailed explanation of cell changes during various stages of plant preparation for cryopreservation and subsequent regrowth.

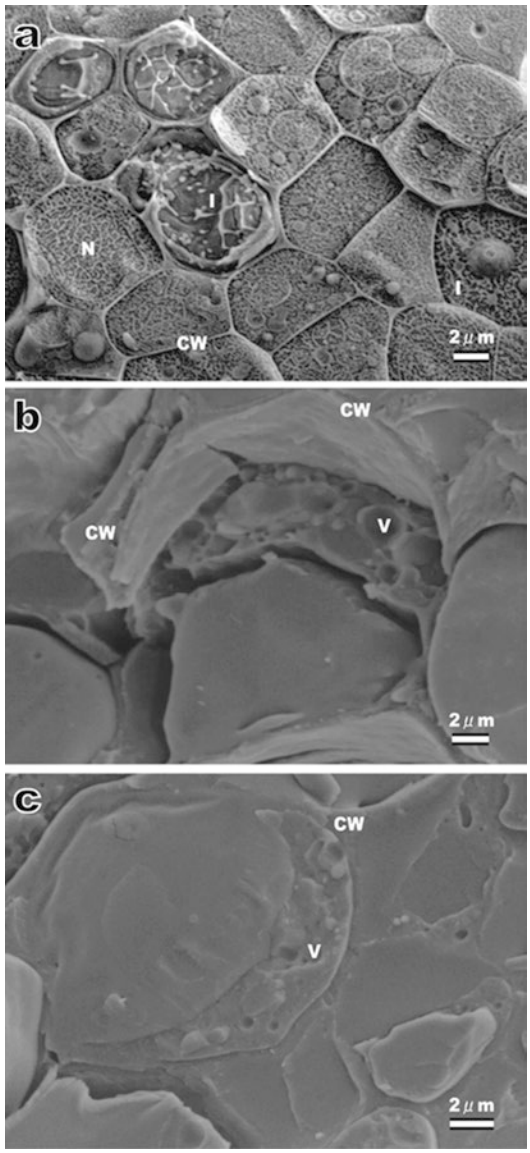

Fig. 19.5 Cryo-scanning electron micrographs showing the structure of *Gentian scabra* meristematic cells. (**a**) Meristematic cells were cryofixed using liquid propane after cold hardening (5 °C for 20 days) and preculture (0.3 M sucrose at 5 °C for 1 day). (**b**) Meristematic cells were cryofixed using liquid propane after the LS treatment. Cold hardening (5 °C for 20 days), preculture (0.3 M sucrose at 5 °C for 1 day), and LS treatment (25 °C for 20 min). (**c**) Meristematic cells that were cryofixed using liquid propane after the PVS2 treatment. Cold hardening (5 °C for 20 days), preculture (0.3 M sucrose at 5 °C for 1 day), LS treatment (25 °C for 20 min), and PVS2 treatment (25 °C for 20 min). I (ice crystals), CW (cell wall), and V (vesicles in cytoplasm) show slight depression from the fracture surface due to slight etching. Bars mean 2 μm

19.5 Application of Cryopreservation Techniques to Specialized Collections

There are many specialized collections in Japan. The Japan National Bio Resource Project has implemented the collection, preservation, and provision of bioresources and the development of related technologies. For some of these specialized bioresources, cryopreservation techniques are being developed.

The liverwort (*Marchantia polymorpha* L.) is one of the key model plants in evolutionary developmental studies, and an increasing number of transgenic and mutant lines of this liverwort have been established. As a case study, a simple cryopreservation protocol using aluminum cryoplates for in vitro grown liverwort gemmae is described. The successive steps of the D cryoplate procedure of liverwort are as follows (Tanaka et al. 2016b). Precultured gemmae are transferred into wells (1.5 mm diameter) of cryoplates (No. 2, Niino and Yamamoto 2017) and embedded in alginate beads by applying a drop of 3% sodium alginate solution. Then, calcium solution is poured dropwise on the section of the cryo-plate for 15 min, achieving complete polymerization of the alginate beads. The encapsulated gemmae on the cryo-plates are soaked in LS (1.0 M sucrose and 2.0 M glycerol) for 30 min. The gemmae are air-dried for 120 min under laminar airflow in a clean hood airflow (0.4–0.6 m s^{-1}). Then, the cryo-plates are placed in cryotubes and placed in LN (−196 °C) or a −80 °C freezer. For regeneration, the cryo-plates retrieved from LN are soaked in 1.0 M sucrose solution at 23 °C for 20 min. Then, gemmae are transferred onto fresh solid gemmae medium without removing the alginate gel and grown (Fig. 19.4). Using this procedure, the cryopreserved gemmae showed a 100% regeneration. This protocol does not require any plant growth regulators such as abscisic acid and takes only 1 h to complete after 1 day of preculture. Furthermore, gemmae treated as described above but then air-dried for 120 min can be stored at −80 °C for at least 1 year without a significant decrease in survival rate, which is

convenient for most laboratories that have a −80 °C freezer but not a LN container for long-term storage. These preservation techniques for *M. polymorpha* should increase their availability to the research community.

D cryo-plate protocol was successfully used for an in vitro collection (polyploidy) of chrysanthemum shoot tips (Tanaka et al. 2016a). Cold-hardened and precultured in vitro shoot tips are placed on a cryo-plate with ten wells (No. 3, oval shape and length of 2.5 mm, a width of 1.5 mm) and embedded in calcium alginate gel. Osmoprotection is performed by immersing the cryo-plates for 30 min at 23 °C in LS (1.0 M sucrose +2.0 M glycerol). The shoot tips are dehydrated by placing the cryo-plates in the air current of a laminar flow cabinet for 90 min. Cooling is performed by placing the cryo-plates in uncapped cryotubes, which are immersed in LN. For rewarming, shoot tips attached to the cryo-plates are rewarmed by immersion in Petri dish containing 1.0 M sucrose solution at 25 °C (Fig. 19.4). Regrowth of cryopreserved chrysanthemum cv. "Seikodaijin" shoot tips using the D cryo-plate protocol was 96.7%. This procedure was applied to seven additional chrysanthemum lines. The average regrowth of vitrified shoot tips of the seven lines was 83.6%, ranging from 66.7% to 100% (Tanaka et al. 2016a).

The cryopreservation techniques described here could contribute not only to the preservation of PGRs but also to the preservation and maintenance of standard, mutant, and transgenic lines of experimental collections and to the preservation of endangered bryophyte species after appropriate optimization.

19.6 Conclusions

Cryopreservation should be considered as a backup to field collections to safeguard against loss of plant genetic resources (Reed 2008). There are many plant species and different propagule types of these species that require development of specific cryopreservation protocols. Variation in relation to sensitivity to physical damage and variation in relation to low temperature and cryoprotectant toxicity need to be considered when developing cryogenic protocols. Currently a range of cryopreservation techniques have been developed such as DV, V cryo-plate, and D cryo-plate. In these protocols, specimens to be cryopreserved are immersed directly into LN on aluminum foil strips or cryo-plates, removal from LN to rewarming solution results in a high level of plant regrowth (Fig. 19.6). This means high regrowth can be obtained by ultrarapid cooling and subsequent warming, even though the water content of specimen is not at an optimal level (Niino and Valle-Arizaga 2015). However, in vitrification method and EV, cooling and warming of specimens are performed using cryotube. Although fine tuning of the protocols is required for successful application to specific plant species and lines, these procedures are facilitating cryostorage of plant genetic resources. Key to cryopreservation is that explants must be in an optimal physiological and morphological state for vigorous regrowth after LN exposure (Engelmann et al. 2008; Sakai et al. 2008).

To further improve procedures, the behavior of cells and water in tissues during the process of plunging samples into cryogens and subsequent warming requires further study. Cryo-SEM observation demonstrated in vivo that there is no apparent ice crystal formation in cells of shoot tips when properly treated with PVS2 and subsequently cooled with cryogens. In addition, many cells of shoot tips exhibited considerable plasmolysis and formation of small vesicles within cells after PVS2 treatment. In contrast, shoot apices without PVS2 treatment contained many large ice crystals throughout cells after cooling with LN, resulting no survival after warming. These results show there is a direct connection between intracellular ice formation and cell death under cryopreservation conditions. Thus, optimal dehydration by PVS2 is essential for cell survival after vitrification at the temperature of LN. Hypertonic treatment with PVS2 will result in osmotic dehydration of cells and an increase in viscosity of cytoplasm, which is a prerequisite for vitrification occurring during cooling (Fahy et al. 1984). It is well known that the propensity of vitrification for water depends on both solute

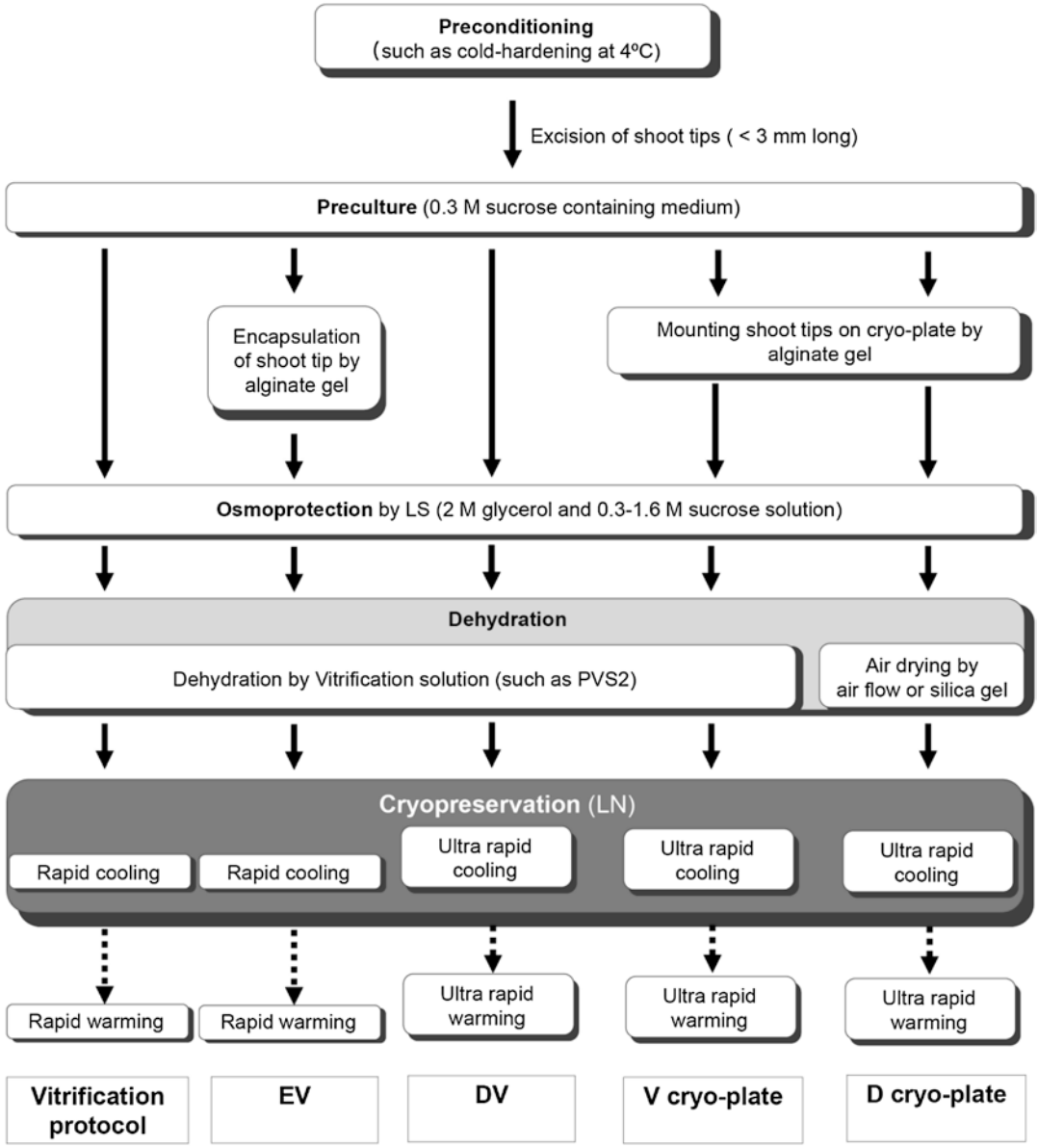

Fig. 19.6 A flowchart of Vitrification EV, DV, V cryo-plate and D cryo-plate protocols

concentrations and cooling rate of the system. In addition, if small ice crystals form during cooling, these ice crystals will grow sufficient to cause severe injury in cells during subsequent warming.

We summarized advantages of some cryopreservation protocols (Table 19.3). Choice of the protocol may depend on the laboratory situation, cost, sensitivity of explant to PVS, and/or staff skills (Reed et al. 2004). An advantage of the DV, V cryo-plate, and D cryo-plate protocols for cryopreservation is that they promote ultrarapid cooling and warming rates. With these cryopreservation protocols, a higher regrowth of the cryopreserved specimens has been obtained after rewarming because of the ultrarapid cooling and warming steps reduced the damage to explants during the cryopreservation process (Yamamoto

Table 19.3 Summarized advantages of cryopreservation protocols

Protocol	Advantages
Vitrification	The shoot tips are dehydrated by PVS2, immersed in LN, and rewarmed in 2 ml cryotube. The sophisticated and valuable protocol. No special equipment needed. Simple and fast procedures. Small shoot tips treated are floating or suspended in solutions. Need careful control of dehydration time by PVS2 to prevent injury by chemical toxicity or excess dehydration. Manipulation of many samples at the same time is difficult. Moderate regeneration rate
EV	The encapsulated shoot tips are dehydrated by PVS2, immersed in LN and rewarmed in 2 ml cryotube. Much flexibility for handling of large amount of materials. The time scale for all steps is much broader. No special equipment needed. Slow dehydration rate of the shoot tips by encapsulation of alginate gels, resulting considerably wide range of dehydration by PVS2. No direct contact to toxic chemical components and LN. Require handling each bead several times, sometimes bead become fragile. Moderate regeneration rate
DV	The dehydrated shoot tips by PVS2 are transferred to a drop of PVS2 on a strip of aluminum foil and immersed into LN directly; in the rewarming step, the strip derived from LN is thawed in diluting solution directly, facilitating their ultrarapid cooling and warming. The volume of shoot tips and alginate gel are becoming uniform. Need skillful manipulation and include cumbersome steps such as osmo-protective and dehydration treatments, transfer of samples on aluminum foil strips, and transfer of foil strips in cryotube. High regeneration rate
V cryo-plate	The shoot tips are attached on the cryo-plate by alginate gel. The osmoprotection, dehydration by PVS2, immersion in LN, and rewarming steps are manipulated by moving the cryo-plate. Handling of the procedure is quick. Ultrarapid cooling and warming. The volume of shoot tips and alginate gel are becoming uniform. The window of optimal dehydration duration by PVS2 is broad. Easy and simple procedure but needs a little practice with mounting of shoot tips on the cryo-plate. High regeneration rate
D cryo-plate	The shoot tips are attached on the cryo-plate by alginate gel. The osmoprotection, dehydration by air flow, immersion in LN, and rewarming steps are manipulated by moving the cryo-plate. Handling of the procedure is easy and quick. Ultrarapid cooling and warming. The window of optimal dehydration duration by air flow is broad. Simple procedure but needs a little practice with mounting of shoot tips on the cryo-plate. Larger specimens can be used. Available for species, which are sensitive to PVS2. High regeneration rate

et al. 2012b; Niino et al. 2013). In this chapter efficient and practical protocols for cryopreservation of PGRs and specialized plant collections have been described. The protocols were applied to a wide array of plant species including wild and multi-ploid species, and they can easily be optimized with minor modifications of standard procedures. These three protocols efficiently complement each other and appear highly promising to facilitate large-scale cryo-banking of PGRs in genebanks.

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20

Applications of Freezing and Freeze-Drying in Pharmaceutical Formulations

Ken-ichi Izutsu

Abstract

Freeze-drying is a popular method to prepare pharmaceutical formulations containing structurally complex active ingredients and drug delivery system carriers. The solidification performed at lower temperature significantly improves storage stability of proteins, peptides, antibiotics, vaccines, and liposomes, which are marginally stable in aqueous solutions. Individual components of the freeze-drying process (freezing, primary drying, secondary drying), however, expose proteins to various stresses. Certain excipients, including disaccharides (e.g., sucrose, trehalose) and amino acids, can be added to protect the proteins and supramolecular drug delivery systems against physical stress associated with freezing and storage by substituting the molecular interactions provided by water molecules. Some excipients embed the active ingredients in glass-state solids with low molecular mobility, thereby reducing chemical reactivity. Thus, the use of appropriate excipients and process control is important to protect proteins during freeze-drying. This chapter describes the applications of freeze-drying in the pharmaceutical production process, mainly focusing on formulation and process optimization for protein therapeutics.

Keywords

Pharmaceutical formulations · Freeze-drying · Protein · Stabilization · Excipient

Abbreviations

ADC	Antibody–drug conjugate
API	Active pharmaceutical ingredient
BSE	Bovine spongiform encephalopathy
CJD	Creutzfeldt-Jakob disease
DDS	Drug delivery system
DSC	Differential scanning calorimeter
LiCl	Lithium chloride
MS	Mass spectrometry
NaCl	Sodium chloride
OD	Orally disintegrating
PAT	Process analytical technology
PVP	Polyvinylpyrrolidone
T_g	Glass transition temperature
$T_{g'}$	Glass transition temperature of maximally freeze-concentrated solute

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20.1 Introduction

Increasing application of structurally complex active molecules (e.g., recombinant proteins, peptides, polynucleotides) and supramolecular drug delivery systems (e.g., liposomes) in pharmaceutical products emphasizes the importance of formulations that allow storage and distribution of products while retaining their desired quality. Many protein formulations, including therapeutic antibodies, are administered via injections or infusions due to their lower oral bioavailability. Although injection-ready formulations are the preferred choice, many protein pharmaceuticals have high propensity for chemical (deamidation, hydrolysis, and disulfide bond cleavage) and physical (perturbation of higher-order structures and aggregation) degradation in aqueous solutions even at refrigerated conditions (2–8 °C) and are not stable enough to endure storage for 1–3 years required for commercial products (Table 20.1) (Lai and Topp 1999; Manning et al. 1989). Preserving the integrity of protein pharmaceuticals is important because such changes not only decrease their therapeutic effectiveness but may also induce severe immunogenic reactions in some patients (Moussa et al. 2016).

Preserving the therapeutic effectiveness of protein pharmaceuticals is primarily achieved using two approaches: modifying the protein structure using protein engineering (e.g., altering amino acid residues) and optimizing their environment through formulation and process design. Protein engineering techniques are usually applied during the early stages of product development, which can also affect the safety and efficacy of the products (Furman et al. 2015). The choice of formulation type (e.g., solidification), excipient composition, packaging, process variables, and storage conditions are important steps in the following development stages (Carpenter et al. 2002).

20.2 Freeze-Drying of Pharmaceutical Proteins

Freeze-drying (lyophilization) is widely used to prepare solid formulations for providing sufficient storage stability to intrinsically unstable proteins and other structurally complex pharmaceuticals (Kasper et al. 2013; Liu and Zhou 2015; Nail et al. 2002; Wang 2000). Approximately 30–40% of protein formulations, including three of the ten highest selling drug products worldwide in 2016, have at least one freeze-dried formulation that are reconstituted before their clinical use (Remicade, Enbrel, and Herceptin). The low processing temperature does not induce severe degradation of proteins that occurs during drying by other methods (e.g., heating). The solidification of proteins and their surrounding molecules decreases the magnitude of most physical and chemical changes that occur during storage. The removal of water is particularly important for maintaining antibody–drug conjugates (ADCs) using a hydrolysable linker (Clavaud et al. 2016). The superior product stability achieved using lyophilization may also contribute to reducing the risk of drug shortage caused by troubles in upstream processes, particularly therapeutic proteins that cannot be substituted by other pharmaceuticals. This stabilization overcomes several obstacles of freeze-drying, including the higher production costs and need for the reconstitution of products before clinical use. The number of frozen soluble products is limited due to the difficulties in temperature control during delivery and storage, although freezing of aqueous solutions is a popular method for storing bulk proteins. The storage of frozen protein solutions at temperatures above −10 °C and/or repetitive freeze–thaw cycles often significantly deactivate the protein products.

20.3 Optimization of Freeze-Drying Process

20.3.1 Freezing of Aqueous Solutions

The development of protein pharmaceuticals requires careful formulation and process designing to overcome various chemical and physical changes to which many proteins are susceptible during freeze-drying and storage (Fig. 20.1) (Arakawa et al. 2001). The freeze-drying of phar-

Table 20.1 Cause of protein aggregation and corresponding stabilization strategies

Cause	High/low pH	High concentration, low ionic strength	Ice–liquid interface	Air–liquid, container–liquid interface	High temperature	Chemical denaturants
Occur mainly in:	Solution, solid	Solution	Solid	Solution, frozen solution, solid	Solution	Solution
Occurs mainly during:	Storage	Process, storage	Process	Process, distribution	Process, distribution	Rare
Protein conformation	Native/altered	Native (self-association)	Partially altered	Altered	Altered	Highly altered
		Partially altered				
Intermolecular interactions	Covalent/noncovalent	Noncovalent	Noncovalent	Noncovalent	Noncovalent	Noncovalent
Reversibility upon dilution	Low (covalent)	High (self-association)	Intermediate	Low	Low	Low
	Intermediate	Intermediate				
Remaining native structure epitope	High	High	High	High	Intermediate	Low
Required effect of excipients	Reducing chemical reaction	Shield hydrophobic protein surface	Substitute water	Reduce exposure to surface	Stabilize protein conformation	Stabilize protein conformation
Typical excipients for stabilization	pH modifier, disaccharide	pH modifier, nonionic surfactant, L-arginine	Disaccharide, amino acid, nonionic surfactants	Nonionic surfactant	Disaccharide, amino acid	Disaccharide, amino acid
Reference	Costantino et al. (1994), Ejima et al. (2007)	Shire et al. (2004)	Costantino et al. (1998)	Shieh and Patel (2015)	Richardson et al. (2000)	Kishore et al. (2012)

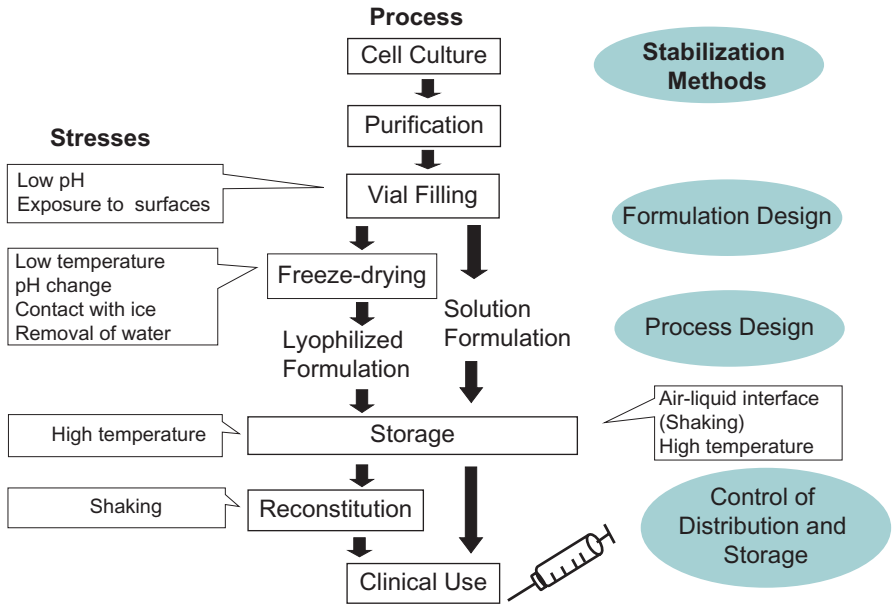

Fig. 20.1 Stresses and stabilization methods of aqueous and freeze-dried protein formulations

maceuticals is usually performed using a shelf-type lyophilizer composed of a product chamber (often with trays on the shelves), refrigeration system, vacuum system, condenser, and control system (Nail et al. 2002). Most freeze-dried formulations are manufactured under aseptic conditions to achieve the required sterility and to avoid protein damage caused by terminal sterilization methods (e.g., exposure of products to high temperature). Lyophilization involves three steps: freezing, primary drying (ice sublimation), and secondary drying (removal of water from the solid phase). The following section describes the standard methods, stress, and new process approaches in freeze-drying amorphous protein formulations.

In the first step of the process, the glass vials containing protein solutions, partially closed with rubber caps, are transferred to the lyophilizer shelves. Then, the shelves are slowly cooled down to −30 to −50 °C, which induces freezing of the aqueous solutions inside the glass vials. The ice growth continues until the highly increased viscosity of the remaining concentrated solution phase (70–80% w/w), present in narrow passages surrounding the ice crystals, loses the apparent molecular mobility. The active pharmaceutical ingredients (APIs) and excipients in the initial solutions possess varied propensities to crystallize [e.g., mannitol, glycine, sodium chloride (NaCl)] or remain amorphous (e.g., sucrose, dextran) in the freeze concentrate (Chang and Patro 2004). Many proteins remain in the non-crystalline freeze-concentrated phase. The solutes mutually affect their crystallization in frozen solutions (Jena et al. 2016). Cooling speed of the lyophilizer shelves and other process parameters also affect physical state of the excipients in frozen solutions (Cao et al. 2013; Peters et al. 2016).

20.3.2 Effect of Freezing on Protein Structure

Freezing of aqueous solutions often alters protein structures due to the low temperature and the freeze concentration of solutes (Arakawa et al. 2001). Lowering the temperature results in the imbalance of various interactions that maintain the native protein conformation, allowing alterations in protein structures (cold denaturation) in ways similar to those of thermal denaturation (Privalov 1990). A large increase in the local concentration of proteins and surrounding excipients (e.g., salts) due to ice growth may also adversely affect the higher-order protein structure and/or induce chemical reactions in the reactive resi-

dues. The crystallization of pH buffer components, such as disodium hydrogen phosphate, alters local pH of the freeze concentrate, which often affect protein stability (Murase and Franks 1989). The exposure of proteins to ice–solution interfaces also induces changes in the protein conformation (Strambini and Gonnelli 2007). The effect of stress at the interface is often more apparent in lower-concentration protein solutions. Protein molecules at the solid surface are also more liable to physical and chemical changes upon storage compared to those buried in the solid (Devineni et al. 2014).

20.3.3 Primary Drying

Primary drying is initiated by reducing the chamber pressure. The removal of ice during the process slowly lowers the interface between ice and solid layers (sublimation interface). During ice sublimation, the protein molecules are kept in the concentrated mixture phase in appropriate processes. The primary drying segment often takes several days due to the slow ice sublimation at low temperatures and perturbation of the water vapor transition through microporous solid layers above the sublimation interface. A failure in the primary drying (e.g., physical collapse) can result in loss of the extremely expensive bulk proteins manufactured by complex processes (Goshima et al. 2016a) (Fig. 20.2). Thus, achieving both rapid ice sublimation and production of microporous "cake" solids is a major challenge in the freeze-drying of noncrystalline solute systems. To achieve shorter process time, the use of larger vials that keep the solution depth below 1 cm is recommended. The product temperatures are determined by the balance between heat provided by conduction, convection, radiation, and that removed by ice sublimation during the primary drying.

20.3.4 Physical Collapse During Primary Drying

Although higher product temperatures allow faster ice sublimation, the primary drying at above glass transition temperature of maximally freeze-concentrated solute (T_g') may induce loss of the microporous solid structure (physical collapse) from the sublimation interface due to low viscosity of the concentrated solute phase and accompanying ice melting (Pansare and Patel 2016). The lowest product temperature that allows physical collapse of the solid is termed collapse temperature (T_c). Although physical collapse does not directly damage the protein structure during the freeze-drying process, many resulting solids are not commercially acceptable due to their inferior appearance and the risk of lower storage stability due to higher residual water content.

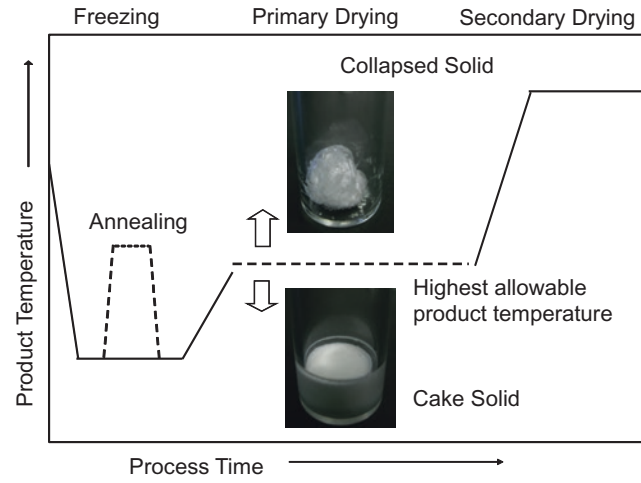

Fig. 20.2 Effect of temperature on physical integrity of freeze-dried solids

20.3.5 Physical Characterization and Process Analytical Technology (PAT) Application for Primary Drying

Rapid primary drying process which maintains the microporous solid structure can be achieved by maintaining the product at temperatures slightly lower than key temperatures (T_g', T_c) of the particular systems. Physical characterization of frozen solutions provides information regarding T_g' and T_c. A freeze-drying microscopic system can be used to indicate the temperature above which the collapse phenomenon occurs in the frozen solution (T_c). The thermal analysis of frozen solutions using differential scanning calorimeter (DSC) helps in determining T_g' of the noncrystalline solute system and crystallization process of some solutes. Many noncrystalline excipients have an intrinsic T_g' that is observed as a change in heat capacity in the thermograms of single-solute frozen solutions. Many saccharides have a T_g' between −45 and −40 °C (monosaccharides) and −36 and −30 °C (disaccharides such as sucrose and trehalose) according to thermal analysis of frozen solutions. Many proteins exhibit no apparent thermal transition in the single-solute frozen solution, while the extrapolation of T_g' profiles of protein and disaccharide mixture solutions suggests that the intrinsic T_g' of frozen protein solutions is approximately −10 °C. Many frozen solutions show T_c at temperatures several degrees higher than T_g' of the corresponding solutions. During primary drying, the products are controlled indirectly by adjusting the shelf temperature and chamber pressure to keep them below the highest allowable product temperatures (T_g', T_c) of the particular system. This approach, which avoids physical collapse, is very different from that in the lyophilization of food products, which is often performed at a product temperature much higher than T_g', allowing local collapse of the heterogeneous solids.

Appropriate transition to the secondary drying segment confirming the endpoint of ice sublimation is also important. The speed of ice sublimation and amount of ice remaining after sublimation vary between vials depending on their ice crystal size and position on the lyophilizer shelves. Premature transition to secondary drying induces melting of the frozen solution layer which remains at the bottom of some vials due to excessive heating. Thus, the primary drying duration is usually set much longer than that predicted from the information obtained by traditional monitoring techniques such as using thermocouples dipped in some vials. PAT tools that provide the product temperature at sublimation interface (manometric temperature monitoring) or air composition in the drying chamber help determination of the primary drying segment endpoint.

20.3.6 Approaches for Efficient Drying Process

Several new techniques that achieve faster primary drying have also been reported. Aggressive freeze-drying is a relatively simple method that holds the product at a temperature slightly above T_c during the primary drying step, forming partially but not fully collapsed solids. Some reports have shown the typical appearance of acceptable and nonacceptable dried solids as well as causes for the process differences (Patel et al. 2017; Patel and Pikal 2009). Inducing larger ice crystals by intentional ice nucleation in the cooling process (controlled nucleation) and heat treatment of frozen solutions (Ostwald ripening during annealing) are promising methods for achieving faster water vapor transition through the ice-sublimed upper phase during the primary drying (Geidobler and Winter 2013; Lim et al. 2018). Conventional gradual cooling of lyophilizer shelves induces heterogeneous nucleation of ice at different temperatures between the vials, resulting in small ice crystals and a solid layer with minute pores that perturb the water vapor transition. Induction of large and uniform ice crystals is considered to allow faster ice sublimation (Beech et al. 2015).

20.3.7 Secondary Drying

Slow heating of the shelf to 25–40 °C and maintaining it for several hours under reduced pressure during the secondary drying segment remove

residual water in the microporous solids (usually <1%, w/w). The removal of residual water increases the glass transition temperature (T_g) of the noncrystalline solids. Rapid heating of the lyophilizer shelf during the early stages of secondary drying may induce shrinkage of the lower T_g amorphous solids containing higher residual water. At the end of secondary drying, the vials are usually closed under reduced pressure on the lyophilizer shelf.

20.3.8 Protein Stability During Secondary Drying and Storage

The removal of hydrating water exposes the protein molecules to further stress in the secondary drying segment. Studies using FTIR and MS suggested lyophilization-induced partial structure changes in many proteins (Chang and Pikal 2009; Iyer et al. 2016). Many of the structurally perturbed protein molecules return to their native structure upon reconstitution in water, but inter- and/or intramolecular interactions between exposed hydrophobic residues often induce their aggregation and other irreversible physical changes. Storage of lyophilized proteins induces various chemical changes, including oxidation and hydrolysis. Less exposure of the interior amino acid residues by co-lyophilization with excipients that retain the protein conformation throughout the process confers better chemical stability to the protein during storage (Cleland et al. 2001).

20.4 Optimization of Formulations

20.4.1 Formulation Design

The optimization of the composition of API and excipients allows efficient process and better stability of freeze-dried formulations. Many pharmaceutical freeze-dried protein formulations contain a stabilizer, pH modifier, and tonicity modifier as excipients. Appropriate formulations depend on a variety of factors, including the intrinsic characteristics of the particular protein, amount required, route of administration, and intended market. For instance, the globally distributed products are more likely to experience harsh environmental conditions, such as extreme temperatures, high humidity, and shaking. The increasing popularity of prefilled syringe formulations that allow self-injection poses new challenges in the manufacturing of freeze-dried proteins (Korpus et al. 2016). Understanding the function of excipients during the process and storage, as well as their underlying mechanisms, should facilitate the rational designing of the protein formulations. Most products use excipients that have already been used in approved pharmaceutical formulations and/or have sufficient safety information to avoid performing additional safety experiments.

Protein concentration in the initial solution is another factor that determines product stability. The required therapeutic dose and applicable injection volume (often <1.5 mL) usually determine the protein concentration in the formulations. Some monoclonal antibodies formulated as high-concentration solutions (>50 mg/mL). Reducing the solution viscosity and risk to protein aggregation by noncovalent interactions are major challenges in the formulation developments (Shire et al. 2004). Contrarily, proteins in low-concentration formulations (<0.1 mg/mL) tend to lose their biological activity as a result of conformational changes that occur at various interfaces.

20.4.2 Cryoprotectants and Lyoprotectants

Many freeze-dried protein formulations contain excipients to protect the proteins from stress during processing and storage. While many polyols are known to protect proteins in aqueous solutions, only some with appropriate physical properties serve as popular stabilizers in freeze-drying. Protein stabilizers can be roughly categorized into three groups based on the phase in which they protect the proteins: aqueous solutions, frozen solutions, and solids. Various saccharides, sugar alcohols, and amino acids protect the higher-order structure of proteins in aqueous

solutions through preferential exclusion which raise the thermal denaturation temperature observed in DSC analysis. They are also considered to protect proteins in frozen solutions through the thermodynamic mechanism. Excipients that protect proteins in frozen aqueous solutions are called cryoprotectants, whereas those that stabilize proteins during freeze-drying are called lyoprotectants.

20.4.3 Stabilization by Water Substitution and Glass Embedding Mechanisms

Two major mechanisms, such as water substitution and embedding in glass-state solids, protect proteins against the stress caused by the removal of surrounding water molecules during the drying phase and subsequent storage (Arakawa et al. 2001; Mensink et al. 2017). Some saccharides and sugar alcohol molecules substitute water molecules that maintain the native protein conformation through intermolecular hydrogen bonding. Multiple studies have indicated a requirement of approximately similar mass ratio of saccharides to proteins in order to fulfill the water-substituting interactions. Maintaining the native conformation in solids also leads to better storage stability of the proteins by preventing the exposure of chemically reactive residues usually buried within the native structure (Cleland et al. 2001). Some small polyols and sugar alcohols (e.g., glycerol, sorbitol) show additional protein-stabilizing effects in the disaccharide-based amorphous solids by providing water-substituting interactions and by filling the molecular-level spaces in the noncrystalline solids (Bellavia et al. 2014). Excess addition of the small polyols, however, may destabilize the proteins due to increasing molecular mobility of the system by their plasticizing effects. Larger saccharide molecules (e.g., dextran) do not form sufficient water-substituting interactions with proteins because of steric hindrance (Tonnis et al. 2015).

Table 20.2 Application of crystallizing and amorphous bulking agents in lyophilized protein formulations

	Crystal	Amorphous
Purpose	Bulking agent	Stabilizer, bulking agent
Popular excipients	Mannitol, glycine	Sucrose, trehalose
Physical stability of solids	Stable	Metastable
Effect on protein stability during:		
Freeze-drying	No contribution	Protect structure by water substitution
Storage	No contribution	Reduce chemical reaction by glass embedding
Possible problem	Protein inactivation, release of crystallization water	Physical collapse, shrink

Izutsu et al. (1993) and Chang and Patro (2004)

Crystallization of some excipients (e.g., mannitol) and accompanying spatial reordering of the molecules deprive them of the protein-stabilizing interactions (Izutsu et al. 1993) (Table 20.2).

Some saccharides form glass-state noncrystalline solids with low molecular mobility that embed protein molecules, thus protecting them from physical and chemical changes during storage. Saccharides and polyols have distinct intrinsic transition temperatures in single-solute frozen solutions (T_g') and dried solids (T_g). Larger saccharide molecules usually show higher T_g' and T_g. Monosaccharides and some small alcohols (e.g., sorbitol), which have lower physical stability in the concentrated phases of frozen solutions and dried solids, are not suitable for use as primary stabilizers in freeze-dried formulations. The use of reducing sugars is not recommended due to their possibility for Maillard reactions with proteins during storage. Both API and excipients should be in a mixed molecular noncrystalline state for protein stabilization in the water substitution and glass embedding mechanisms.

20.4.4 Stabilization Using Sucrose and Trehalose

Sucrose and trehalose are the most commonly used stabilizers in recent freeze-dried protein formulations because of their protein-stabilizing effects by water substitution and glass embedding. Approximately 1:1 mass ratio of the disaccharide to protein fulfills the protein-stabilizing molecular interactions. The choice between sucrose and trehalose as a stabilizing agent depends on the target proteins and intended clinical use. Freeze-drying with sucrose often shows greater retention of the protein secondary structure in FTIR than that with trehalose. However, the sucrose-base amorphous solids have higher risk of physical changes (e.g., shrinking) or chemical degradation upon storage at higher temperature due to the lower T_g and formation of reducing sugars by hydrolysis. Trehalose confers better physical stability to noncrystalline dried solids that have higher T_g and minimizes crystal growth by holding the residual water in the form of a dehydrate molecule (Kawai et al. 1992). However, trehalose can crystallize during long-term storage of the frozen solutions.

20.4.5 Use of Multiple Excipients

The freezing step in lyophilization concentrates proteins and multiple excipients into narrow spaces surrounded by ice crystals. Most protein and excipient combinations are freeze-concentrated into mixed molecular noncrystalline phase that shows single T_g' in thermal analysis. Since addition of higher concentration disaccharide lower T_g' and T_g of the protein-disaccharide mixture, using disaccharides at the concentration essential for the structural stabilization would enable faster primary drying (high T_g' and highest allowable product temperature) and ensure better storage stability of the resulting solids (higher T_g). Some combinations of protein and polymer excipients in an aqueous solution separate into different freeze-concentrated phases that are rich in one of the components (amorphous–amorphous phase separation) (Izutsu and Kojima 2000). Understanding heterogeneity of the component compositions in a solid should assist further improvement of the formulation design (Twomey et al. 2015). Crystallization of certain excipients also separates the freeze-concentrated solutes into multiple phases different in composition and physical states. Mannitol and glycine are often used as crystallizing bulking agents that hold protein molecules and amorphous stabilizers (Sundaramurthi and Suryanarayanan 2010). Some formulations contain a combination of crystallizing and amorphous excipients in the physically stable microporous solids.

20.4.6 pH and Tonicity Modifiers

Many injectable protein formulations have a moderately acidic to neutral pH in solution. Several pH buffer systems, including sodium phosphate and histidine hydrochloride, are used in commercial formulations (Zbacnik et al. 2017). The choice of appropriate pH and pH modifiers based on the properties of the particular protein and clinical application is a necessary step in the development of freeze-dried formulations. To prevent the undesirable pH change in the freeze concentrate and resulting solids, the use of crystallizing pH modifiers is often avoided in formulations (Pikal-Cleland et al. 2002). The use of pH modifiers at lower concentrations, satisfying the required buffer capacity, also supports better stability of the freeze-dried products because the buffer salts often serve as plasticizers in the saccharide-based systems. Many injectable solution formulations contain NaCl as a tonicity modifier. Other excipients may be preferable for freeze-dried formulations because the high crystallization propensity and plasticizer effect of NaCl on the disaccharide-based noncrystalline phase (lowering of T_g') often destabilize the co-lyophilized proteins. Lower concentration of the salts (e.g., NaCl, LiCl) improves stability of some proteins during lyophilization (Goshima et al. 2016b).

20.4.7 Amino Acids

Amino acids have been used as pH modifiers (L-histidine) or crystallizing bulking agents (glycine) in freeze-dried protein formulations. Some amino acids show apparent protein-stabilizing effects during the freeze-drying process and following storage (Stärtzel 2016). Some of them improve protein stability in combination with saccharides (Forney-Stevens et al. 2016). The effects of amino acids, particularly those not observed in saccharides, are gathering increasing attention in the development of freeze-dried protein formulations. For example, the ability of L-arginine to prevent protein aggregation has been actively studied to improve storage stability of solution and/or freeze-dried monoclonal antibody formulations (Izutsu et al. 2005). The use of appropriate counter ions enables design of amino acid-based freeze-dried solids that have desirable physical properties (Moussa et al. 2016).

20.4.8 Surfactants and Polymers

Some surfactants and polymers protect certain proteins during the freeze-drying process. Surfactants, such as polysorbate 80, are often added to aqueous solution and lyophilized protein formulations to decrease the structural changes at the ice–solution interface and/or adsorption to the container. The effect of surfactant is often more apparent in lower-concentration protein solutions as the ratio of molecules under stress at the interface decreases with increasing protein concentration.

Some soluble polymers, such as PVP and dextran, prevent the dissociation of protein subunits during freeze-thawing due to exclusion volume effects (Anchordoquy et al. 2001). In freeze-drying, the combination of polymers and smaller saccharides provides both water-substituting interactions and glass-state solids (Allison et al. 2000). Some proteins without particular biological effects (e.g., albumin) also protect therapeutic proteins by serving as a pH modifier that also reduces protein denaturation at the interfaces. However, their use in the development of new pharmaceutical formulations is limited because of contamination risks (e.g., bovine spongiform encephalopathy and/or Creutzfeldt–Jakob disease). Some chemicals that bind specifically to particular proteins (ligands) may also protect the protein structure against physical stress.

20.4.9 Laboratory-Scale Freeze-Drying of Proteins

Many strategies for production-scale lyophilization can be applied for small freeze-drying batches (e.g., total solution volume <100 mL) using a conventional laboratory lyophilizer without appropriate shelf temperature control. The selection of higher T_g' excipients that allow primary drying at higher temperatures and setting conditions for faster ice sublimation (e.g., use of larger volume vials) may assist in simplifying the freeze-drying process. Formulations containing relatively higher concentrations of the protein concentrations may contribute in reducing the risk of physical collapse.

20.5 Freeze-Drying of Other Pharmaceuticals

20.5.1 Liposomes

Liposomes have been used as a potent drug delivery system (DDS) for API molecules (e.g., AmBisome). Freeze-drying is a popular method of protecting liposomes against chemical (hydrolysis and oxidation) and physical (aggregation, membrane perturbation, and API erosion) changes occurring during the storage of aqueous suspensions. The dehydration of liposomes apparently improves storage stability, whereas the stress induced by ice growth and low temperature during freeze-drying induces membrane structure perturbation that leads to API erosion and/or liposome aggregation (Chen et al. 2010). Disaccharides protect liposomes through the water substitution (interaction with polar phos-

pholipid head groups) and the glass embedding mechanisms. The stabilization of freeze-dried liposome pharmaceuticals require the optimization of multiple parameters in the formulation and process, such as lipid composition, solution pH, type of stabilizers, and shelf temperature controls (Sylvester et al. 2018). Freeze-drying is also applied to prepare other DDS system formulations such as polymeric nanoparticles (Fonte et al. 2016).

20.5.2 Vaccines and Small-Molecule APIs

Certain structurally complex antibiotics and vaccines have been formulated as freeze-dried solids because they are only marginally stable in aqueous solutions and/or heating at higher temperatures (Hansen et al. 2015). Antibiotics crystallize or remain amorphous in frozen solutions depending on their intrinsic property, formulation, and thermal history. Crystallized APIs usually exhibit better storage stability than noncrystalline solids because of their limited mobility in the crystal lattice. Maintaining a relatively simple composition, mainly focusing on improving the chemical stability and adjusting tonicity, is often preferred for freeze-dried formulations involving the low-molecular-weight APIs. Freeze-drying is also applied to prepare OD tablets that disintegrate and dissolve rapidly with small amount of water on the tongue (Lai et al. 2014). The effect of freeze-drying on the stability of vaccine formulations and methods used to stabilize them largely depend on the constituent antigens. Information regarding the stabilization of therapeutic proteins and liposomes should be useful for the rational designing of formulations and processing (Remmele et al. 2012).

20.6 Conclusion

Freeze-drying is an important process that facilitates the distribution of various complex pharmaceuticals. Extensive studies on the stabilization of recombinant proteins have provided information on the choice of excipients and underlying mechanisms that allow rational designing of formulations and freeze-drying process. The combination of protein engineering and formulation technologies may enable the supply of safe and effective pharmaceuticals. Furthermore, this information may be applicable in small-scale drying and/or development of dried foods.

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Control of Physical Changes in Food Products

21

Kiyoshi Kawai and Tomoaki Hagiwara

Abstract

Food is a multicomponent system that mainly comprises protein, carbohydrate, fat, and water. During food processing and preservation, various physical changes (e.g., melting, crystallization, glass transition) occur in food products, affecting their quality. This chapter specifically examines the effect of physical changes on the quality of dry and frozen food products. Dry food products are commonly in an amorphous state. Therefore, glass transition occurs during their dehydration–rehydration processing. To control their texture and physical stability, it is important to elucidate the effects of water contents on the glass transition temperature of dry food products. Frozen foods consist of ice crystals and freeze-concentrated matrix. The formation of ice crystal and the dynamics of ice crystal evolution affect food quality. Therefore control of ice crystals is important for high-quality frozen food. Moreover, because freeze-concentrated matrix consists of solute that are plasticized by the unfrozen water and is in an amorphous state, it can undergo glass transition by freeze concentration. The physical state of freeze-concentrated matrix also strongly affects the stability of food quality during frozen storage.

Keywords

Glass transition · Water plasticizing · Ice crystallization · Dry food · Frozen food

Abbreviations

AFP	Antifreeze protein
DSC	Differential scanning calorimetry
MD	Maltodextrin
T_g	Glass transition temperature
T_g'	Glass transition temperature of the maximally freeze-concentrated phase
TRA	Thermal rheological analysis
W_c	Critical water content

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21.1 Introduction

Food is a multicomponent system that mainly comprises protein, carbohydrate, fat, and water. During food processing and preservation, various

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physical changes (e.g., melting, crystallization, glass transition) occur in food products, affecting their quality. This chapter specifically examines the effects of physical changes on the quality of dry and frozen food products. In the first part, effect of water content on the glass transition temperature (T_g) of dry food products (dry fruits and cookie) and trial to predicting T_g is presented for understanding physical changes of dry food products induced by water sorption. In the second part, physical changes of frozen food products during cooling and frozen storage, such as ice crystallization, freeze concentration, eutectic separation, glass transition, recrystallization of ice crystal, and sublimation of ice, and their effects on quality of frozen food are reviewed.

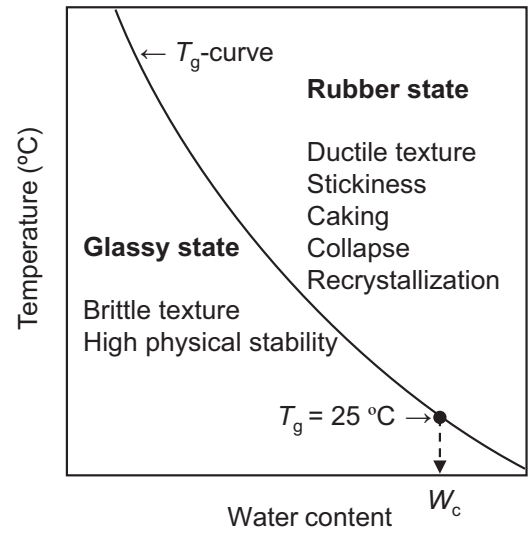


Fig. 21.1 Effect of water content on the T_g (T_g curve) of dry food products. The water content at which T_g is 25 °C is described as the critical water content (W_c)

21.2 Glass Transition of Dry Food Products

21.2.1 Role of Glass Transition in Dry Food Products

Dry food products are commonly in an amorphous state. Therefore, they undergo a glass to rubber transition (glass transition) during dehydration–rehydration processing. This transition involves a physical change between a solid-like (glassy) state and a liquid-like (rubbery) state and causes a drastic change in rheological properties. The temperature at which glass transition occurs is known as the glass transition temperature (T_g). Glass transition can also occur with no change in temperature if the water content changes because T_g of hydrophilic amorphous solids decreases with increased water contents. The water content at which T_g is 25 °C is described as the critical water content (W_c). The glass transition behavior can be described as a T_g curve (effect of water content on T_g), as presented in Fig. 21.1. In the area under the T_g curve, the food product is in a glassy state. Glassy food products have a hard or brittle texture because of their high elasticity (Payne and Labuza 2005a, b). In addition, glassy food products are expected to have greater physical stability than rubber ones because of their low molecular mobility. In the area above the T_g curve, the food products are in a rubber state. Rubbery food products, which have a soft or ductile texture, undergo various physical changes (e.g., stickiness, caking, collapse, sugar crystallization) because of their high molecular mobility (Roos 1995; Jaya and Das 2004; Cano-Chauca et al. 2005; Palzer 2005; Harnkarnsujarit and Charoenrein 2011a, b; Zou et al. 2013). To control their texture and physical stability, it is important to ascertain the T_g curve of dry food products.

21.2.2 Glass Transition of Dry Fruits

Dry fruits include large amounts of low-molecular-weight carbohydrates (e.g., sucrose, fructose, glucose). Because these carbohydrates have a low T_g, dry fruits readily undergo a glass transition because of water sorption.

The T_g of dry fruits is commonly evaluated using differential scanning calorimetry (DSC). Glass transition can be detected as an endothermic shift in a DSC thermogram. However, glass transition observed in the first scanning shows an endothermic peak because of the enthalpy relaxation effect depending on the thermal history of glassy samples (Kawai et al. 2005). Enthalpy

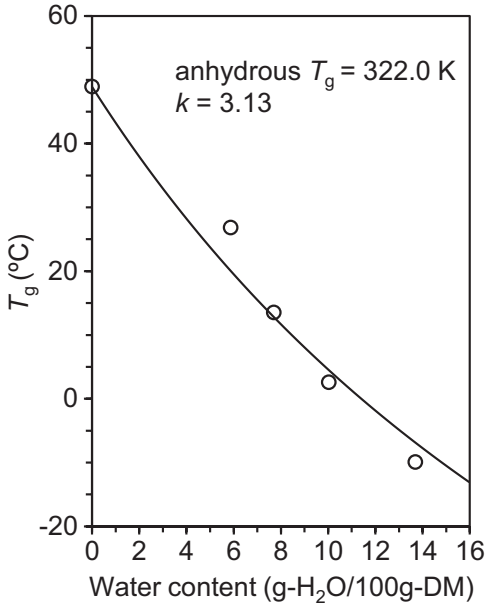


Fig. 21.2 Effect of water content on the T_g of dry mango pulp. The T_g was evaluated using DSC. The solid line was given by the Gordon–Taylor equation with two parameters (anhydrous T_g and k). The anhydrous T_g was evaluated experimentally and k was determined as a fitting parameter

relaxation is a process wherein the excess enthalpy of glass in a non-equilibrium thermodynamic state decreases spontaneously toward its equilibrium value. The T_g value is known to be affected to various degrees by the enthalpy relaxation effect (Haque et al. 2006). To cancel the thermal history of glassy samples on T_g, DSC measurement is taken again after the first scanning. Therefore, T_g can be determined from the onset point of the shift in the second scanning.

Effect of water content on the T_g of dry mango pulp is shown in Fig. 21.2. The T_g decreased with increased water content because of water plasticizing effect. This behavior can be summarized as a T_g curve, as depicted in Fig. 21.2. The solid line represents fitting of the Gordon–Taylor equation (Eq. 21.1) to the T_g data.

$$T_g = \frac{W_s T_{gs} + kW_w T_{gw}}{W_s + kW_w} \quad (21.1)$$

Therein, W_s and W_w, respectively, represent the weight fractions of mango pulp and water. T_{gs} and T_{gw} are T_g for anhydrous mango pulp and water, respectively, whereas k is a constant. T_{gs} was evaluated experimentally. T_{g2} was set to 136 K, as reported in the literature (Johari et al. 1987; Sastry 1999). When k was determined as a fitting parameter, the T_g curve presented in Fig. 21.2 was obtained. The k represents the sensitivity to water plasticizing: higher k is associated with greater T_g depression caused by water sorption. From the T_g curve, W_c can be evaluated.

For low-molecular-weight carbohydrates including polyol, a linear relation is known to exist between anhydrous T_g (°C) and k (Roos 1995) as

$$k = 0.0293 \times \text{anhydrous} T_g + 3.61 \quad (21.2)$$

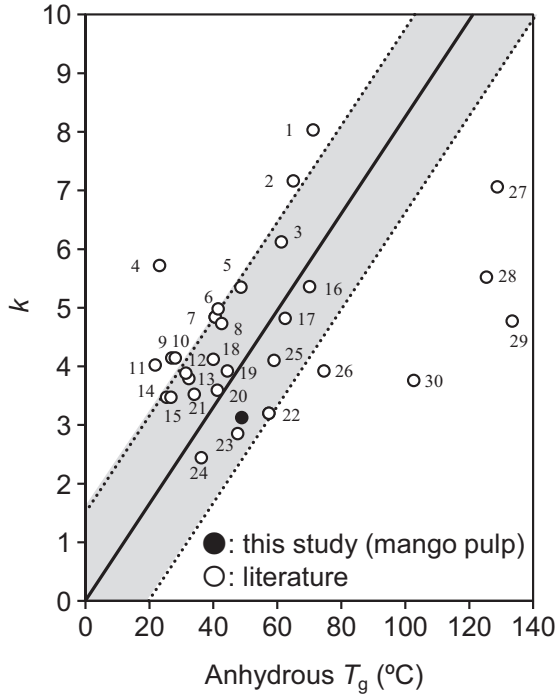
The major constituent of dry fruits is low-molecular-weight carbohydrates. Therefore, similar behavior would be expected in dry fruits. The relation between anhydrous T_g and k for dry fruits of various types (Bai et al. 2001; Fabra et al. 2011; Kasapis et al. 2000; Khalloufi et al. 2000; Kurozawa et al. 2012; Moraga et al. 2004, 2006, 2011; Mosquera et al. 2010, 2012; Oikonomopoulou & Krokida 2012; Silva et al. 2006; Sobral et al. 2001; Sonthipermpoon et al. 2006; Syamaladevi et al. 2009; Telis 2006; Telis & Martínez-Navarrete 2009; Vásquez et al. 2013; Wang et al. 2008; Zotarelli et al. 2017) is presented in Fig. 21.3. Although some outliers (e.g., camu camu, persimmon, plum) were observed, there was a roughly linear relation.

$$k = 0.0825 \times \text{anhydrous} T_g + 0.005 \pm 1.5749 \quad (21.3)$$

As described above, a T_g curve can be described by the Gordon–Taylor equation with two parameters (anhydrous T_g and k). W_c can be evaluated from the T_g curve. Consequently, Eq. 21.3 is expected to be useful for the prediction of T_g depression and physical changes of dry fruits induced by water sorption.

21.2.3 Physical Modification of Dry Fruits Based on T_g

As described above, dry fruits undergo physical deterioration of various types at temperatures higher than T_g. To improve their physical stability,



1. Kiwifruit1 (homogenized tissue) (Moraga et al., 2006)
2. Kiwifruit1 (entire tissue) (Moraga et al., 2006)
3. Banana (Moraga et al., 2011)
4. Chinese gooseberry (Wang et al., 2008)
5. Banana flake (Sonthipermpoon et al., 2006)
6. Kiwifruit 2 (homogenized tissue) (Moraga et al., 2006)
7. Kiwifruit 2 (entire tissue) (Moraga et al., 2006)
8. Raspberry (Syamaladevi et al., 2009)
9. Strawberry (homogenized tissue) (Moraga et al., 2004)
10. Strawberry (entire tissue) (Moraga et al., 2004)
11. Blueberry (Khalloufi et al., 2000)
12. Strawberry (Oikonomopoulou and Krokida, 2012)
13. Mango with maltodextrin (Zotarelli et al., 2017)
14. Apple (Moraga et al., 2011)
15. Mango (Zotarelli et al., 2017)
16. Borojó (Mosquera et al., 2010)
17. Noni (Fabra et al., 2011)
18. Blackberry (Khalloufi et al., 2000)
19. Camu-Camu (Silva et al., 2006)
20. Apple (Bai et al., 2001)
21. Strawberry (Khalloufi et al., 2000)
22. Date (Kasapis et al., 2000)
23. Raspberry (Khalloufi et al., 2000)
24. Blueberry (Vásquez et al., 2013)
25. Strawberry (Mosquera et al., 2012)
26. Grapefruit (Telis and Martínez-Navarrete, 2009)
27. Papaya (Kurozawa et al., 2012)
28. Camu-Camu with maltodextrin (Silva et al., 2006)
29. Persimmon (Sobral et al., 2001)
30. Plum (Telis, 2006)

Fig. 21.3 Relation between anhydrous T_g and k for various types of dry fruits. The solid line is a mean linear relationship. The upper and lower dotted lines are the variation of the mean linear relationship. Most of the data exist in the gray zone. The applied ranges of anhydrous T_g and k were 21–75 °C and 2.0–8.0, respectively

it is important to elevate the T_g of dry fruits by additives with high-T_g materials. Maltodextrin (MD), which has a much higher T_g (100–243 °C) than low-molecular-weight carbohydrates (Goula and Adamopoulos 2008), has been used as the physical modifier of dry fruits. For example, some related reports on it are being used in camu camu (Silva et al. 2006), tomato pulp (Goula and Adamopoulos 2008), grapefruit juice powder (Telis and Martínez-Navarrete 2009), borojó powder (Mosquera et al. 2010), orange juice powder (Goula and Adamopoulos 2010), strawberry (Mosquera et al. 2012), and mango powder (Zotarelli et al. 2017). In an earlier study conducted by the authors (Fongin et al. 2017), the effect of MD addition on the glass transition properties of dry mango pulp was investigated. It was demonstrated that the anhydrous T_g increased with an increase in MD content (Fig. 21.4). A systematic study showed that an abrupt anhydrous T_g change occurred between 60% and 70% MD. Similar observations were also obtained for the T_g change for glucose–MD and maltose–MD mixtures (Kawai and Hagura 2012). These results suggest that amorphous mixtures have heterogeneous molecular dynamics. MD is plasticized by the mango pulp above 70% MD content. Consequently, the anhydrous T_g decreases continually with increasing mango pulp content. In the region, glass transition of the mango pulp–MD mixture will occur cooperatively. Part of the mango pulp is excluded from the mango pulp–MD domain when the MD content decreases to less than 60%. Then, not only a mango pulp–MD domain but also a mango pulp-rich domain (lower anhydrous T_g) is formed in the amorphous system. Consequently, an abrupt anhydrous T_g change can be observed at MD contents of 60–70%.

The k value of dry mango pulp also increased with an increase in MD content. The relation between anhydrous T_g and k obeys Eq. 21.3 below MD content of 60%. By the further addition of MD, the relation between anhydrous T_g

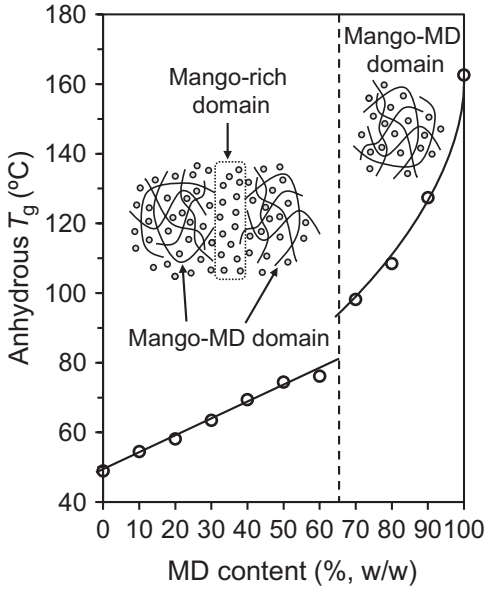


Fig. 21.4 Effect of maltodextrin (MD) addition on the anhydrous T_g of mango pulp. An abrupt anhydrous T_g change occurred between 60% and 70% MD. The suggested turning point is indicated by dotted line. Inserted drawing is a model of heterogeneous molecular dynamics in the amorphous system

and k deviates from Eq. 21.3 (for dry fruits) and approaches Eq. 21.2 (for low-molecular-weight carbohydrates). This can be related to the suggestion described above: the mango pulp-rich domain is lost from the amorphous mixture system at MD contents between 60% and 70%.

21.2.4 Glass Transition Properties of Dry Bakery Products

The T_g of amorphous materials has commonly been evaluated using DSC. However, when a more complex multicomponent system (e.g., a cookie) is subjected to DSC measurement, a continuous thermal response is observed. Also, the endothermic shift associated with the glass transition is overlapped. In addition, because starch-based food intrinsically shows a small and broad endothermic shift, it is difficult to evaluate the T_g from the DSC thermogram. In such cases, thermomechanical approaches such as thermomechanical compression test (TMCT), thermal mechanical analysis (TMA), and dynamic thermal mechanical analysis (DMTA) are useful. For example, T_g values of barley (van Donkelaar et al. 2015), rice (Thuc et al. 2010), dairy powder (Hogan et al. 2010), peas (Pelgrom et al. 2013), chocolate wafers (Payne and Labuza 2005b), and abalone (Sablania et al. 2004) have been found using thermomechanical approaches. An earlier study conducted by the authors (Kawai et al. 2014) established thermal rheological analysis (TRA), which is almost equivalent to TMCT and TMA. The TRA curve shows a clear force drop associated with the glass transition: mechanical T_g can be determined from the onset point. It is confirmed that TRA is an effective tool to evaluate T_g of bakery products including cookies. It is noteworthy that T_g determined by thermomechanical approaches does not always agree with that determined by DSC (Sandoval et al. 2009). Thermal T_g observed by DSC has a clear physical meaning, representing the temperature at which the viscosity is approximately 10^{12} Pa•s (Angell et al. 1994). Mechanical T_g is more sensitive to experimental conditions such as mechanical force, heating rate, and sample quantity (Lacík et al. 2000; Ross et al. 2002; Boonyai et al. 2007).

Cookie dough mainly comprises wheat flour, sugar, butter, and egg. Three types of sugar composition (sucrose alone, sucrose containing 40% trehalose, and sucrose containing 40% sorbitol on a dry weight basis) were used and baked for cookie production. Effect of water content on the mechanical T_g of the handmade cookie samples is presented in Fig. 21.5. The mechanical T_g of the cookie samples decreased with an increase in water content because of water plasticizing effect, similar to the case for dry fruits. The onset point would be independent of water content if the force drop observed in the TRA curve resulting from the melting of fat. The fact that the force-drop point decreased with an increase in water content reveals that the rheological response resulted from glass transition of the cookie samples and not simply by the melting of fat. The water content of cookies is approximately 3–4% under the initial conditions. Consequently, it was noted that normal cookies (sucrose alone) were in a glassy state at 25 °C,

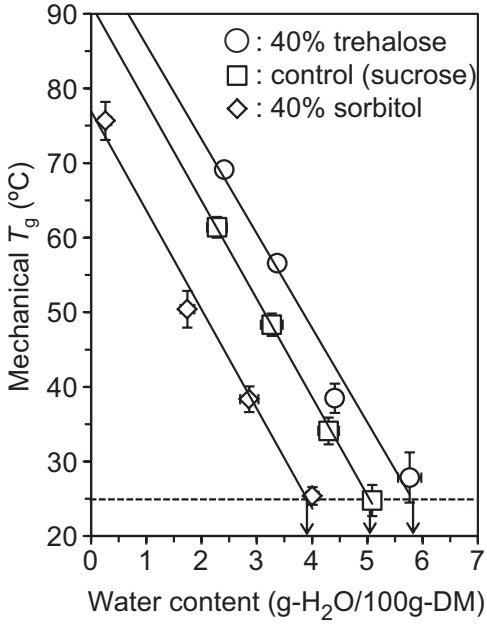


Fig. 21.5 Effect of water content on the mechanical T_g of cookie samples with varying sugar compositions. The mechanical T_g was evaluated using TRA

which explains why they had a brittle texture. When the water content of normal cookies became higher than W_c (5.04 g-H_2O/100 g-DM), they transitioned into a rubber state. Therefore, the brittle texture changed to a ductile one. Trehalose-added cookies had higher mechanical T_g and W_c than normal cookies because trehalose has higher anhydrous T_g (114 °C) than sucrose (62 °C). Consequently, it was concluded that trehalose can improve the physical stability of cookies; trehalose led to the brittle–ductile texture change occurring at higher water content than in normal cookies. By contrast, sorbitol-added cookies had lower mechanical T_g and W_c than normal cookies had because sorbitol has lower T_g (−9 °C) than sucrose. Considering these findings, the sorbitol-added cookies showed ductile texture, even at low water content. This texture is also important to control the cookie texture.

It is noteworthy that anhydrous mechanical T_g for cookies increased with an increase in the T_g for the sugar composition used for cookie preparation. When the anhydrous mechanical T_g for cookies (extrapolated value) was shown against anhydrous T_g for sugar composition (experimental value obtained by DSC), a linear relation (anhydrous mechanical T_g for cookie = 0.3245 × anhydrous T_g for sugar composition +71.62) was identified, which indicates that the mechanical T_g of cookies can be characterized by the T_g of sugar composition. Cookies comprise a continuous glassy sugar or toffee-like matrix containing embedded starch granules, an undeveloped gluten network, and fat (Slade and Levine 1994; Chevallier et al. 2002). Consequently, the physical properties of cookies depend strongly on the sugar composition. Numerous publications describe the T_g of carbohydrate materials and their mixtures. They are expected to be useful for predicting the T_g of cookies.

21.3 Physical Changes of Frozen Food Products During Cooling and Frozen Storage

21.3.1 Ice Crystallization in Frozen Food Products During Cooling

During cooling of food, crystallization of water component occurs after supercooling and nucleation. The size of ice crystal in frozen food strongly affects its quality. Rapid freezing gives smaller ice crystals and even distribution of ice crystals in food. The rate of ice nuclei formation is larger at lower temperatures. Ice crystal growth is most accelerated around the equilibrium ice melting temperature, a zone of maximum ice crystal formation (0 to −5 °C). Therefore more nuclei occur, and ice crystal growth is suppressed when a food is passed quickly through a maximum ice crystal production zone by rapid cooling. Rapid freezing is desirable for most foods. Slow freezing produces larger ice crystals, inhomogeneous locations of ice crystals, shrunken appearance of the microstructure, and usually lower quality than rapid freezing. For cryopreservation of living cells and microorganism, slow freezing might sometimes be preferred (Franks 1985). Rapid freezing usually causes intracellular ice crystal formation, which is lethal for cells. Slow freezing

initiates the formation of nuclei outside cell and extracellular ice crystallization occurs. Furthermore, cells are dehydrated during the process of extracellular ice crystallization. Extracellular crystallization and dehydration of cells suppress intracellular crystallization. Practically speaking, an aid of cryoprotectant such as dimethyl sulfoxide (DMSO), glycerol, and trehalose is needed for successful cryopreservation of living cells and microorganisms. Cryoprotectants permeate into a cell or dehydrate a cell by osmotic pressure action. Consequently, water contents in the cell are reduced, which suppresses intracellular crystallization (Franks 1985).

21.3.2 Freeze Concentration

Upon cooling food products below 0 °C, ice formation and separation of water from food solids occur. Consequently, solute concentration of unfrozen part increases as ice contents increase with declining temperatures. This phenomenon is called "freeze concentration." Generally, a rate of chemical reaction decreases at lower temperatures. However, the chemical or biochemical reaction rate increases sometimes because of lowered temperatures when the effect of increase of reactant by freeze concentration overcomes that from lowered temperatures (Fennema et al. 1973; Franks 1985). The freeze concentration engenders a change of pH or electrolyte concentration in food, which can cause protein denaturation (Fennema et al. 1973; Franks 1985).

Freeze concentration has been used for the concentration of liquid foods such as fruit juices (Deshpande et al. 1982; Bayindirli et al. 1993; Miyawaki et al. 2016), vegetable juices (Miyawaki et al. 2005), and dairy products (Hartel and Espinel 1993). Among the methods of concentrating liquid food, freeze concentration presents several benefits: low energy requirements, low process temperature preventing undesirable chemical and biochemical changes, and minimal loss of flavors and aromas (Ramteke et al. 1993; Liu et al. 1997).

21.3.3 Eutectic Separation

Lowering the temperature continuously, the concentration of freeze-concentrated phase reaches its solubility at last. At this point, the concentration of solute cannot become any higher without increasing the temperature (Franks 1985). When more ice is formed, the amount of dissolved solute is too high and solute crystals start to form simultaneously. This phenomenon is called "eutectic separation." The temperature at which eutectic separation occurs is known as the eutectic point. The eutectic point of sodium chloride is −21.1 °C. Practical eutectic separation probably occurs only rarely in most frozen foods because of the complex nature of food materials (Roos 1995). However, it is well known that lactose in ice cream is often crystallized during frozen storage by supersaturation. It does not crystallize immediately after freezing because of kinetic constraints, but it crystallizes after a certain period of storage. Subsequently, the crystals grow (Roos 1995). When lactose crystals grow so large that they can be detected in the mouth, the smooth texture of ice cream is transformed into a sandy texture: an unacceptable product (Marshall and Arbuckle 2000).

21.3.4 Glass Transition by Freeze Concentration

It is quite often true that the solute does not crystallize at the eutectic point from aqueous solution system within a practically realistic time scale. Consequently, freeze concentration proceeds continuously by lowering the temperature. Finally, the freeze-concentrated phase turns into a glassy state at a specific temperature (T_g'). Ice crystallization ceases at T_g' because the high viscosity of the freeze-concentrated matrix suppresses diffusion of water molecules to the surface of existing ice crystal (Franks 1985; Roos 1995). Frozen food below T_g' is a mixture of the glassy amorphous substance and ice crystals. It is generally acknowledged that the deterioration of frozen food during storage is minimized by

maintaining it below T_g' (Franks 1985; Agustini et al. 2001). It is noteworthy that exposure of a freeze-drying material to temperatures higher than T_g' causes melting of ice crystals and softening of the freeze-concentrated matrix (Roos 1995). Because the freeze-concentrated matrix cannot support its own weight, collapse, reduced water removal rate, and inferior product quality occur (Roos 1995).

21.3.5 Ice Recrystallization During Frozen Storage

Ice crystal size and shape are unstable during frozen storage even if the temperature is constant. They change continuously through the process of "recrystallization," which includes any change in number, size, shape, orientation, or perfection of crystals following the completion of initial solidification (Fennema et al. 1973). Recrystallization proceeds as a result of minimization of surface free energy of the entire crystal phase (Fennema et al. 1973; Hartel 1998). Recrystallization of ice crystals during storage and distribution is a major cause of deterioration in frozen foods, especially frozen desserts. Generally, the recrystallization of ice crystals in frozen foods is characterized as an increase in the mean size of ice crystals (Fennema et al. 1973; Hartel 2001) (Fig. 21.6).

Three recrystallization mechanisms are likely to occur during the conventional storage of frozen food: migratory, isomass, and accretion (Fennema et al. 1973; Hartel 2001). Migratory recrystallization refers to the trend for larger crystals in a polycrystalline system to grow in size at the expense of smaller crystals. Smaller crystals cannot bind their surface water molecules as tightly as larger ones because of the higher curvature and higher surface free energy. Therefore, the water molecules on the surface of smaller crystals tend to transfer to the surface of larger ones through the freeze-concentrated

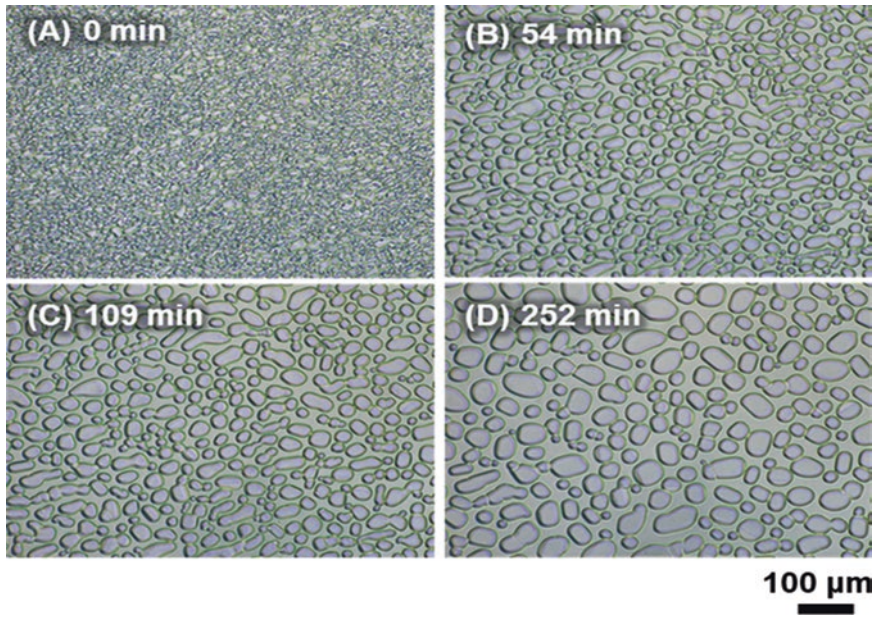


Fig. 21.6 Example of ice recrystallization during storage. Sample, 25% glucose; storage temperature, −10 °C; storage time (**a**) 0 min, (**b**), 54 min, (**c**) 109 min, (**d**) 252 min; observation procedure, 2 μl sample solution enclosed between two coverslips (diameter, 12 mm) was placed on the sample stage to be frozen at −22 °C. After 10 min, the temperature was elevated to the observation temperature (−10 °C) at a rate of 1.5 °C/min. Finally, after the observation temperature was reached, ice crystal images were periodically photographed (Hagiwara et al. 2006)

matrix, engendering the growth of larger crystals and disappearance of smaller ones. A similar process called isomass recrystallization can occur in a single crystal. Consider a single separated crystal with a rough surface. The part of the surface with higher curvature cannot bind surface water molecules as tightly as a smoother surface. As a result, the rougher surface becomes smoother. Accretion recrystallization is the process by which two crystals that are in mutual contact grow together into one larger crystal. Because the contact point has apparently high curvature and because it is not as stable as the rest, neck formation occurs by transportation of water molecules to this region, which engenders growth into one crystal.

When temperature fluctuations occur during storage, the recrystallization process is enhanced by melt–refreeze recrystallization, which is more important for ice cream texture/shelf life in practical situations than during isothermal processes (Hartel 2001).

To describe a change of ice crystal size by isothermal recrystallization, the following two equations have been used.

$$R^3 = R_0^{\,3} + kt \tag{21.4}$$

$$R^2 = R_0^{\,2} + kt \tag{21.5}$$

Therein, R stands for the averaged size of ice crystals, R_0 signifies the averaged size of ice crystals at time $t = 0$, and k denotes the isothermal recrystallization rate constant. The recrystallization process in ice cream and its model solutions can be described well by Eq. 21.4 (Sutton et al. 1996; Hagiwara et al. 2006; Klinmalai et al. 2017). Equation 21.5 was applied for ice recrystallization in frozen beef (Bevilacqua and Zaritzky 1982).

Recrystallization of ice crystals in frozen foods has been studied extensively. The group of Zaritzky et al. studied the recrystallization of frozen beef and its model system (Bevilacqua and Zaritzky 1982; Martino and Zaritzky 1988, 1989). The effects of storage temperature, temperature fluctuation, sweeteners, and stabilizers on the recrystallization rate of ice crystals in ice cream have also been reported (Donhowe and Hartel 1996a, b; Hagiwara and Hartel 1996; Miller-Livney and Hartel 1997).

Table 21.1 Sample sugar solutions. Values of ice content and concentration of freeze-concentrated matrix were calculated by using literature values of freezing point depression versus solute concentration (Young and Jones 1949; Young et al. 1952; Young 1957; Lide 2003)

Sample	Storage temperature(°C)	Freeze-concentrated matrix conc. (%)	Ice content (%)
21.8% maltose	−4.4	40.0	45.4
22.45% sucrose	−4.6	41.1	45.4
18.5% glucose	−5.8	34.0	45.5
25.0% sucrose	−5.8	45.8	45.5
22.45% fructose	−8.0	41.1	45.4
28.6% sucrose	−8.0	52.4	45.4
25.0% fructose	−10.0	45.8	45.5
25.0% glucose	−10.0	45.9	45.4

The ice contents of all samples were set almost equal to omit the detailed discussion about effect of ice content on recrystallization rate (Hagiwara et al. 2006)

To elucidate recrystallization phenomena, it is necessary to comprehend not only the correlation between production conditions and recrystallization rate constant but also the molecular-level mechanisms accounting for different recrystallization rates. A better understanding of the molecular mechanisms of recrystallization is expected to facilitate the systematic prediction and control of recrystallization behavior for various frozen foods. Recently it has been recognized that the concept of water mobility is useful for predicting and controlling the recrystallization rate in frozen foods (Ablett et al. 2002; Hagiwara et al. 2006, 2009; Klinmalai et al. 2017). Reportedly, recrystallization rates in a series of frozen saccharide solutions (Table 21.1) increased concomitantly with increasing the diffusion coefficients of water component in the freeze-concentrated matrix. A direct relation was found between the recrystallization rate and the diffusion coefficient (Hagiwara et al. 2006, 2009) (Fig. 21.7). This

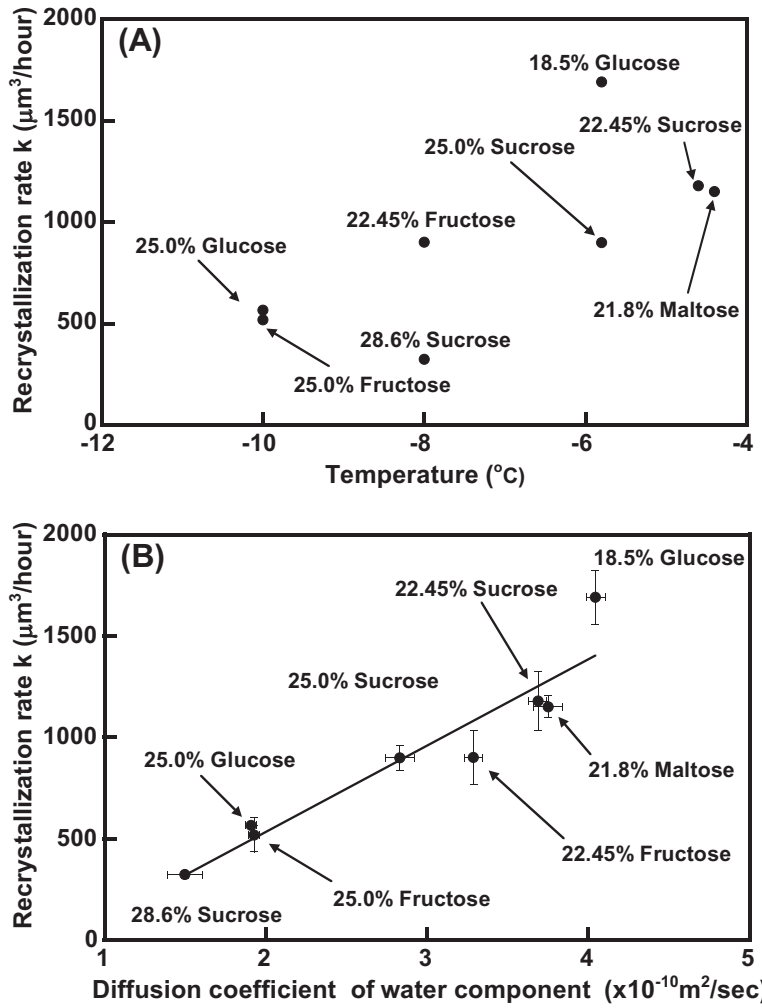


Fig. 21.7 Plots of recrystallization rate constant K of ice crystals in various frozen saccharide solutions as a function of (**a**) temperature and (**b**) diffusion coefficient of water component in a freeze-concentrated matrix. Sample saccharide solutions are listed in Table 21.1. The recrystallization rate constant K of ice crystals was obtained by using Eq. 21.4 (Hagiwara et al. 2006). The diffusion coefficient of water component was measured by pulse field gradient stimulated gradient echo proton NMR method (Hagiwara et al. 2006)

relation indicates that the self-diffusion coefficient of water component in freeze-concentrated matrix is a useful parameter for predicting and controlling recrystallization. The ^{1}H spin–spin relaxation time T_2 of water components in freeze-concentrated matrix also shows good correlation with the recrystallization rate constant (Ablett et al. 2002; Klinmalai et al. 2017).

21.3.6 Effects of Glass Transition on Recrystallization of Ice Crystals

Based on the concept of water mobility in freeze-concentrated matrix, the recrystallization of ice crystals is expected to be strongly suppressed below T_g' because the molecular mobility is strongly restricted. Hagiwara et al. (2005) investigated the recrystallization of ice crystals in a 30% sucrose solution at temperatures of −21 to −50 °C, including temperatures around T_g' (−32 °C). As the storage temperature decreased, a rapid decline in the recrystallization rate was observed between −29 and −35 °C (Fig. 21.8). This result is consistent with the concept of the glass transition of the freeze-concentrated matrix. It is noteworthy that even at −50 °C, at which the freeze-concentrated matrix is regarded as being in glassy state, an increase in the mean crystal size was observed within 20 h storage. This observation suggests that, over a realistic storage period, deterioration by recrystallization might occur, even in the glassy state. In general, it is

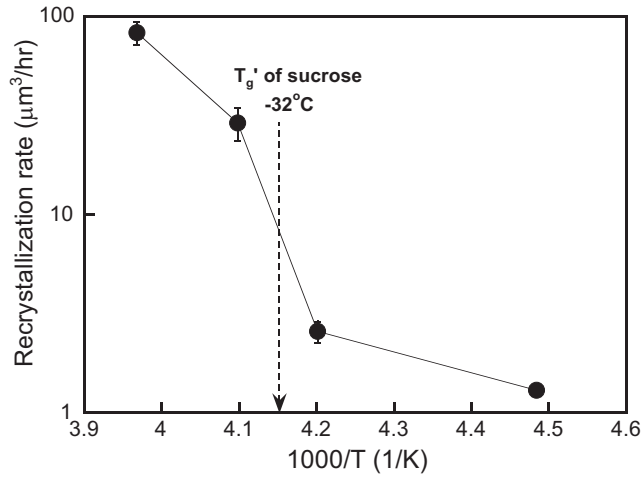


Fig. 21.8 Arrhenius plot of recrystallization rate of ice crystals in frozen 30% sucrose solutions

believed that food in a glassy state is very stable because its molecular motion is restricted severely. However, in the field of polymer science, it is well-recognized that molecular movement leading to macroscopic structural relaxation over a practical period remains below the glass transition temperature because of the non-equilibrium nature of glassy substances (Matsuoka 1992; Yoshida 1995; Tiemblo et al. 2002). Molecular mobility in glassy food and food component carbohydrates with low moisture content has also been investigated recently (Hancock et al. 1995; Urbani et al. 1997; Kim et al. 2003; Hashimoto et al. 2004; Kawai et al. 2005). Regarding frozen food systems, molecular mobility in the freeze-concentrated phase of trehalose (Pyne et al. 2003) and sucrose solutions (Inoue and Suzuki 2005) below T_g' was investigated on the concept of enthalpy relaxation. Molecular motion in a freeze-concentrated solute matrix might be sufficient to cause ice recrystallization over a realistic storage period, even in a glassy state.

21.3.7 Recrystallization of Ice Crystals in the Presence of Antifreeze Protein (AFP)

Recently, many antifreeze proteins (AFPs) have been extracted from a variety of sources (Davies and Hew 1990; Graether et al. 2000; Griffith and Yaish 2004; Regand and Goff 2006; Kawahara et al. 2009). They are now anticipated as additives for suppressing ice recrystallization process. AFPs suppress ice crystal growth by an adsorption–inhibition mechanism (Raymond and DeVries 1977). They inhibit thermodynamically favored ice crystal growth by their adsorption to specific planes of ice crystals (Raymond et al. 1989).

To put AFP into practical use as a recrystallization suppressor, evaluation of their suppression ability is fundamentally important. A typical AFP extracted from polar fish, AFP type I, actually suppresses the recrystallization of ice crystals efficiently, with quite a low concentration (Fig. 21.9) (Hagiwara et al. 2011). The recrystallization rate constant of 33 wt% sucrose solution containing 1 μg/ml AFP type I was about 13% of that of sample without AFP (Table 21.2). No marked reduction of recrystallization rate was observed for samples containing 0.1 μg/ml or 0.01 μg/ml AFP type I.

21.3.8 Sublimation of Ice in Frozen Food Products

When frozen foods are stored without appropriate moisture-proof package, the food surface is dehydrated. Consequently the surface has an opaque appearance called "freezer burn" (Fennema et al. 1973). Freezer burn results from the sublimation of ice on the surface of frozen foods when the water vapor pressure of the ice is higher than that in the environmental air

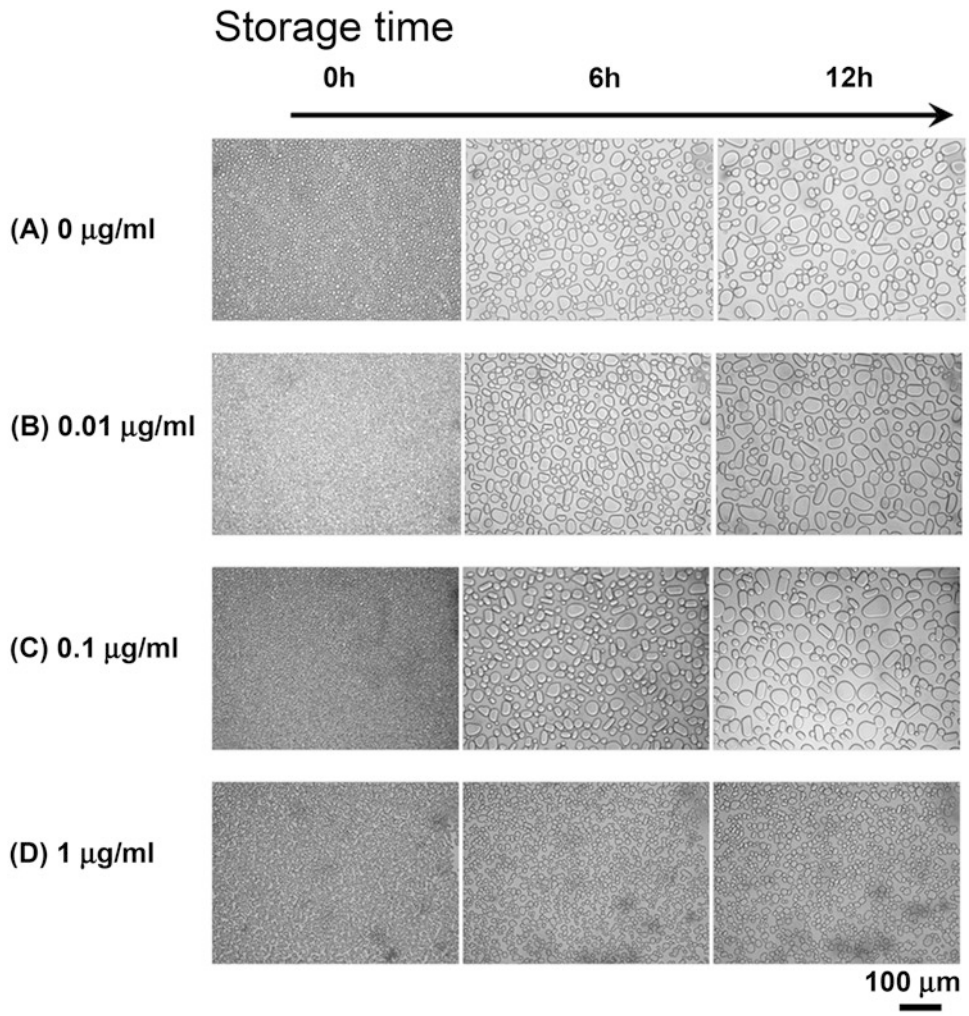


Fig. 21.9 Effects of AFP type I addition on recrystallization of ice crystals in 33% sucrose solution at −10 °C. The concentrations of AFP type I; (**a**) 0 μg/ml, (**b**) 0.01 μg/ml, (**c**) 0.1 μg/ml, (**d**) 1 μg/ml

Table 21.2 Summary of recrystallization rate constants for the samples with different AFP type I concentrations

	0 μg/ml	0.01 μg/ml	0.1 μg/ml	1 μg/ml
Recrystallization rate constant k ($\mu m^3/h$)	186 ± 54	200 ± 58	173 ± 25	24 ± 8

(Fennema et al. 1973). The temperatures of specific parts of the freezer shelf, such as the heat exchanger, are lowest: lower than the stored frozen foods. Therefore, the situation described above normally occurs in most cases. Vapor from the frozen food surface adheres to the surface of the lower temperature part in the shelf of freezer, which is familiar to us as "frost." The sublimation of ice enhances many deteriorative processes such as lipid oxidation, color change, protein denaturation, odorous emissions, and the decline of nutritional value. Therefore, suppression of ice sublimation is necessary for the long-term preservation of frozen foods.

21.4 Conclusion

As reviewed above, melting, crystallization, and glass transition occur in the food products during food processing and preservation and affect their quality. This chapter will help to control and predict the quality of dry and frozen food products. In addition, it is important to understand the adaptation mechanisms of organism in extreme cold and desiccation as seen in the other chapters.

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