J. Plant Biol. (2009) 52:19–26
DOI 10.1007/s12374-008-9006-6

REVIEW

Molecular Basis of Disease Resistance Acquired through Cold Acclimation in Overwintering Plants

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Received: 19 November 2008 / Revised: 9 December 2008 / Accepted: 9 December 2008 / Published online: 16 January 2009

Abstract Plants require substantial resistance against freezing and pathogens for overwintering. These two traits are acquired through cold acclimation. In contrast to freezing tolerance, molecular basis of disease resistance acquired through cold acclimation is poorly understood. Recent studies have suggested that pathogenesis-related (PR) proteins that are secreted into the apoplast during cold acclimation are responsible for the disease resistance. Interestingly, some of the cold-induced PR proteins display both antifungal and antifreeze activities, suggesting a dual function in protecting plants from overwintering stresses. The signaling pathway for cold-induced disease resistance is currently unknown but can be independent of pathogen-induced defense mechanisms.

Keywords Antifreeze protein · Apoplast · Cold acclimation · Snow mold · Pathogenesis-related protein

Abbreviations

ABA	abscisic acid
AFP	antifreeze protein
CA	cold-acclimated
ET	ethylene
JA	jasmonic acid
NA	non-acclimated
PR	pathogenesis-related
SA	salicylic acid

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Introduction

Overwintering plants, such as winter wheat and forage grasses acquire resistance against harsh winter conditions by sensing temperature downshift in late autumn. This process is called cold acclimation or winter hardening. Cold acclimation is accompanied by diverse physiological and morphological changes. Through cold acclimation plants acquire ability to survive freezing upon exposure to subzero temperatures. In the areas with heavy and persistent snow, plants need to increase disease resistance to survive from the attack of pathogens known as snow molds. It is also important for plants to induce dormancy during cold acclimation in order to reduce consumption of reserve carbohydrates during the winter under snow. Therefore, cold acclimation is considered a complex adaptation mechanism of plants to winter environment.

Snow molds are a group of psychrophilic fungi that infect and damage plants under snow cover [17, 25]. Resistance to snow mold is a quite important trait for crop winter survival. In fact, in heavy snow regions such as northern Japan, breeding program is more focused on improving snow mold resistance than freezing tolerance. In contrast to the extensive studies regarding pathogen-induced defense responses in plants [24], research on low-temperature-induced disease resistance is quite limited. In this review, we present current sketch of physiological and molecular aspects of disease resistance acquired through cold acclimation.

Overwintering Requires Disease Resistance

Snow Mold Diseases

Snow mold diseases are caused by psychrophilic fungi called snow molds that grow and infect perennial or overwintering annual plants during winter season (Fig. 1)

Fig. 1 **a** Winter wheat field damaged by snow mold diseases. The white patches are the killed plants. **b** Field-grown winter wheat plant that was killed by a snow mold (*Sclerotinia borealis*). Black sclerotia are formed on the surface of dead leaves

[17, 25]. Snow molds cause serious damage on economically important plants such as winter cereals, forage and turf grasses, floriculture plants, and conifers. The major snow mold diseases for winter cereals include pink snow mold (*Microdochium nivale*), *Sclerotinia borealis*, gray snow mold (*Typhula incarnata*), and speckled snow mold (*Typhula ishikariensis*). These fungi favor near-freezing temperatures, and dark and humid conditions for growth and plant infection. Snow molds invade plants through stomata or cuticle and slowly promote infection and propagation over several months. In northern temperate and boreal regions where long persistent snow covers field, snow molds are the most serious diseases of winter wheat and turf grasses. Snow mold fungi initiate host-plant infection in late autumn or early winter when the soil is not yet frozen. During the winter period (November through early March), snow mold fungi proliferate and spread in host tissues under dark and humid conditions that are brought about by thick snow cover.

Disease Resistance Induced by Cold Acclimation

Overwintering plants acquire not only freezing tolerance but also disease resistance against pathogens during cold acclimation [28, 31, 35]. In field-grown wheat, increase in snow mold resistance is correlated with the drop in air temperature from October over December [28]. In a controlled environment, wheat markedly increases snow mold resistance upon cold treatment (2-6°C) for a week (Fig. 2). The cold-induced disease resistance is considered non-specific or basal resistance. Barley and meadow fescue (*Festuca pratensis*) acquire resistance against *Bipolaris sorokiniana* through cold acclimation [31]. Similarly, rape increases resistance against the pathogen, *Phoma lingam* upon cold treatment [31]. Barley acquires the disease resistance quickly after 6-week cold acclimation, however meadow fescue acquires the resistance progressively during cold acclimation. These data suggested that overwintering plants have diverse expression mechanisms of disease resistance in response to cold.

Defense-related Proteins are Induced by Cold Acclimation

Cold-induced Defense Related Proteins

Pathogenesis-related (PR) proteins were initially identified as host-encoded polypeptides that were synthesized in response to tobacco mosaic virus infection in tobacco. Subsequently, PR proteins have been identified from a wide variety of plant species and are now classified into 17 families based on amino acid sequence similarity and enzymatic activity (Table 1) [24]. Many of PR protein

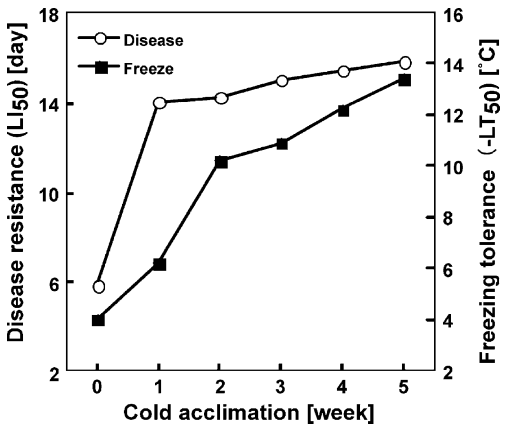

Fig. 2 Low temperature induces disease resistance. Both freezing tolerance (LT_{50}) and resistance against pink snow mold (LI_{50}) increase during cold acclimation. LI_{50}: days of incubation to achieve 50% mortality. LT_{50}: 50% killing temperature. Data were modified from Nakajima and Abe [28]

Table 1 Cold-inducibility of pathogenesis-related (PR) proteins

Family	Cold inducible isoform	Properties	Plants	Reference
PR-1	+	unknown	Wheat	Gaudet et al. [7]
PR-2	+	β-1,3-glucanase	Rye, wheat	Gaudet et al. [7]; Hon et al. [16]
PR-3	+	Chitinase type I, II, IV, V, VI, VII	Rye, wheat	Gaudet et al. [7]; Hon et al. [16]
PR-4	Nd	Chitinase type I, II	–	–
PR-5	+	Thaumatin-like	*Arachis hypogaea*, chestnut, rye, wheat	Dave and Mitra [20]; Garcia-Casado et al. [6]; Hon et al. [16]; Kuwabara et al. [22]
PR-6	+	Proteinase-inhibitor	Barley, chestnut	Garcia-Casado et al. [6]; Pernas et al. [30]
PR-7	Nd	Endoproteinase	–	–
PR-8	Nd	Chitinase type III	–	–
PR-9	+	Peroxidase	Wheat	Gaudet et al. [7]
PR-10	+	Ribonuclease-like	Dandelion, Douglas fir, mulberry, peach, western white pine, yellow-fruit nightshade	Ekramoddoullah et al. [3, 4]; Liu et al. [23]; Ukaji et al. [36]; Wisniewski et al. [38]; Xu et al. [42]
PR-11	Nd	Chitinase, type I	–	–
PR-12	+	Defensin	Peach, wheat	Koike et al. [21]; Wisniewski et al. [39]
PR-13	+	Thionin	Wheat	Gaudet et al. [8, 9]
PR-14	+	Lipid transfer protein	Cabbage, chestnut, wheat	Gaudet et al. [8, 9]; Hincha et al. [14]
PR-15	Nd	Oxalate oxidase	Barley, pepper	Jung et al. [20]; Pearce et al. [29]
PR-16	Nd	Oxalate oxidase like	–	–
PR-17	Nd	Unknown	–	–

classes have been shown to contain anti-microbial activities and are thought to play a role in pathogenesis-induced defense mechanisms. Interestingly, recent researches revealed that a variety of PR proteins were induced by low temperature (Table 1). The transcript of PR-1, -2, -3, -9, -13 and -14 accumulate during cold acclimation in winter wheat [7–9]. PR-2, -3, -5 proteins accumulate in the apoplastic fraction of cold-hardened winter rye [16]. Apoplastic PR-5 proteins induced during cold acclimation have also been identified in winter wheat [22], *Arachis hypogaea* [2], and chestnut [6]. PR-10 in western white pine [3], Douglas fir [4], dandelion [42], peach [38], and mulberry [36] are seasonally regulated and rapidly accumulate in the winter. Winter wheat *TAD1* [21] and peach *PpDfn* [39], which encode a plant defensin (PR-12), are induced during cold acclimation. The transcripts of PR-14 are induced by cold-treated cabbage [14], chestnut [6], and pepper [20]. Barley *blt4.9*, encoding a PR-14, is induced during cold acclimation [29] as well as pathogen infection [27]. These researches suggested that most classes of PR protein are involved in plant response to low temperature.

Antifreeze Protein

Antifreeze proteins (AFPs) that bind to hexagonal ice crystal to prevent its growth have been identified in some organisms that can tolerate subzero temperature [19]. AFPs were first identified in the blood of marine teleost fishes from the Antarctic sea. Binding of AFP to ice crystals results in depression of the freezing point without affecting the melting point of a solution, the process which is known as thermal hysteresis. The freezing point-depression occurs non-colligatively and is 500 times greater than that of colligative salts on a molar basis. AFPs bind to the prism face of hexagonal ice crystal and affect the morphology of ice crystals, creating bipyramidal ice crystals in solution (Fig. 3).

Plants exposed to subzero temperatures undergo extracellular freezing. Extracellular freezing begins with the formation of ice crystals in the apoplast. AFPs in the apoplast bind to the ice crystal and inhibit its growth. Since plant AFPs show substantially lower thermal hysteresis activity than animal AFPs, the main function of plant AFPs are considered to be recrystallization-inhibiting activity which is known as another typical activity of AFPs [12]. Growth of ice crystals within the apoplast can cause a critical damage on the cells. AFPs produced in the apoplast can inhibit fusion of ice crystals (Fig. 3).

Plant Antifreeze and Antifungal Proteins

The first plant AFP was reported in 1992 [37]. Thermal hysteresis activity was detected in several plant extracts sampled during the autumn and winter in northern Indiana, USA. Since the activity was absent in the samples collected during the summer, it was suggested that the thermal

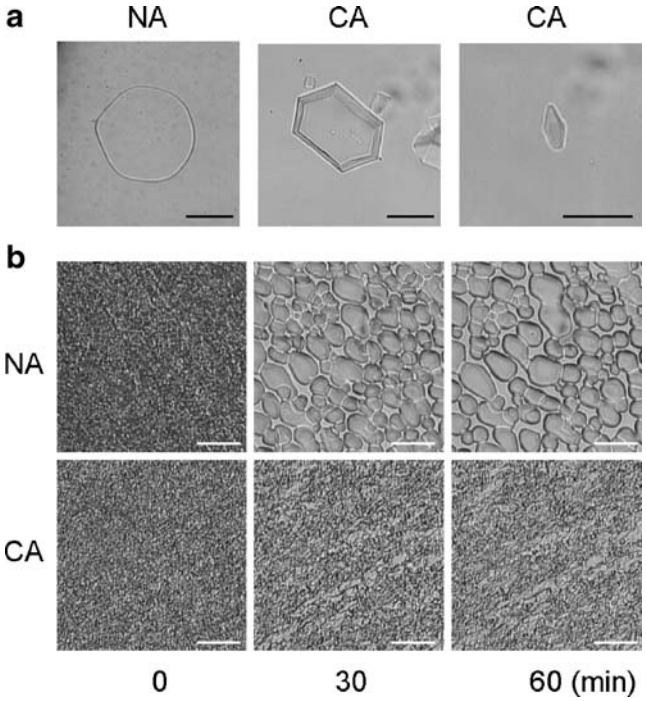

Fig. 3 Antifreeze activities of wheat apoplast proteins. **a** Assays of antifreeze activity by ice crystal morphology. Leaf apoplastic protein fraction from non-acclimated plants (NA) lacks antifreeze activity. Without binding activity of the proteins, ice crystals grow to round and flat shapes. Apoplastic proteins from cold-acclimated (21 days) plants (CA) bind to the prism face of the crystal, resulting in creation of hexagonally shaped crystals. *Scale bars* 50 μm. **b** Assay of the recrystallization inhibition activity. Protein fractions containing 30% sucrose were flash-frozen at −20°C and then held isothermally at −6°C. Crystal growth at 30 and 60 min shows that the CA sample has recrystallization inhibitory activity. *Scale bars* 50 μm

hysteresis activity was induced during cold acclimation [37]. Stem extract from bittersweet nightshade (*Solanum dulcamara*) exhibited the highest thermal hysteresis activity (0.45°C) as well as recrystallization inhibition activity. Griffith et al. [11] also found that an apoplastic protein fraction from cold-acclimated winter rye leaves contain AFPs with a molecular size ranging from 13 to 36 kDa [15]. These proteins showed sequence similarity to PR proteins such as β-1,3-glucanase-, chitinase-, and thaumatin-like protein. Among them, the chitinase-like protein actually exhibited enzymatic activity, indicating the protein is a dual, antifreeze and antifungal, function [16]. Intriguingly, similar chitinase and β-1,3-glucanases that are induced in the apoplast upon snow mold infection did not exhibit antifreeze activity [16] (Table 2).

Carrot AFP (DcAFP) is a glycoprotein with a secretory signal and shares 56% sequence identity with kiwi polygalacturonase-inhibitor [26, 41]. The C-terminal three quarters of DcAFP are made up with leucine-rich repeat sequence (~24 amino acids) that is suggested to form an ice-binding domain [49]. Polygalacturonase inhibitor is considered as a PR-like protein since polygalacturonase is involved in pathogen infection to plants [24]. However, the inhibitory activity of DcAFP can be inactive, since DcAFP was unable to inhibit polygalacturonase activity from *Aspergillus niger* [41].

The AFPs from bittersweet nightshade mentioned above were further characterized and identified. The gene for the 67-kDa AFP (*sthp-64*) showed high sequence similarity to *WRKY1* from tobacco and *ZAP1* from *Arabidopsis*. STHP-64 contains a unique ten-tandem repeat of a 13-mer motif that is absent in WRKY1 and ZAP1. Since such a repetitive sequence is commonly found in animal AFPs, it was predicted for ice-binding site [18]. In addition, STHP-64 also binds to the ZAP-1C sequence that is the optimal binding sequence for *Arabidopsis* ZAP1, indicating that STHP-64 has a potential transcription factor function.

All previously reported plant AFPs are inducible during cold acclimation and most of them are antifungal or PR proteins that are secreted into the apoplast. Although STHP-64 and PCA60 were accumulated exceptionally within the cells, these proteins may play roles in preventing lethal intracellular freezing by binding to ice nuclei occurred intracellularly [12]. Most plant AFPs are considered to decrease the damage of plant cells to inhibit ice growth by recrystallization of ice crystal occurred in the apoplast [12]. As previously mentioned, overwintering plants need to acquire snow mold resistance as well as freezing tolerance to survive in the winter. Apoplast is the interface between plant cells and stresses associated with freezing and pathogen infection. Therefore, it seems a reasonable strategy for overwintering plants to produce bifunctional antifreeze and antifungal proteins in the apoplast.

Diversity of Cold-induced Defense-related Proteins

Defense-related proteins such as antifungal proteins occupy a relatively large portion of cold acclimation-induced genes. In fact, a subtraction screening revealed that the percentage of defense-related genes accounted for 17% of genes that were induced during cold acclimation of winter wheat [8, 9]. A defensin gene (*TAD1*) was shown to be inducible during cold acclimation of winter wheat [21]. The TAD1 protein contained a signal sequence and was secreted into the apoplast. Recombinant TAD1 protein (rmTAD1) displayed growth inhibition of the pink snow mold, *Microdochium nivale*, and *Pseudomonas cichorii*. However, rmTAD1 did not show antifreeze activity [21]. A cold-inducible multidomain cystatin showed an antifungal activity against pink snow mold but failed to show antifreeze activity [1]. Similarly, a thaumatin-like protein (WAS-3) that accumulated in the apoplast during cold acclimation inhibited growth of pink snow mold but failed to show antifreeze activity [22]. Judging from these results, it was suggest that overwintering

Table 2 Plant antifreeze proteins

Plant	Protein/gene name	M.W. (kDa)	Homology	Reference
Ammopiptanthus mongolicus	–	28	None	Griffith and Yaish [12]
Bittersweet nightshade (*Solanum dulcarnara*)	STHP64	67	WRKY transcription factor	Huang and Duman [18]; Urrutia et al. [37]
Bittersweet nightshade	STHP47	47	Chitinase-like protein	Huang and Duman [18]; Urrutia et al. [37]
Bittersweet nightshade	STHP29	29	Class I chitinase-like protein	Huang and Duman [18]; Urrutia et al. [37]
Carrot (*Daucus carota*)	DcAFP	36	Polygalacturonase inhibitor protein	Meyer et al. [26]; Worrall et al. [41]
Douglas fir (*Pseudotsuga menziesii*)	–	27	Class I chitinase-like protein	Zamani et al. [48]
Peach (*Prunus persica*)	PCA60	60	Dehydrin	Wisniewski et al. [40]
Perennial ryegrass (*Lolium perenne*)	LpAFP	29	None	Sidebottom et al. [32]
Winter rye (*Secale cereale*)	ScGlu-1	33.6	β-1,3;1,4-glucanase	Hon et al. [16]; Yaish et al. [43]
Winter rye	ScGlu-2	33.4	β-1,3-endoglucanase	Hon et al. [16]; Yaish et al. [43]
Winter rye	ScGlu-3	33.4	β-1,3-endoglucanase	Hon et al. [16]; Yaish et al. [43]
Winter rye	CHT9	35	Class I endochitinase	Hon et al. [16]; Yeh et al. [44]
Winter rye	CHT46	28	Class II endochitinase	Hon et al. [16]; Yeh et al. [44]
Winter rye	–	25	Thaumatin-like protein	Hon et al. [16]
Winter rye	–	16	Thaumatin-like protein	Hon et al. [16]
Winter wheat (*Triticum aestivum*)	TaIRI1	27	leucine-rich repeat protein	Tremblay et al. [33]
Winter wheat	TaIRI2	41	leucine-rich repeat protein	Tremblay et al. [33]

plants protect themselves from snow mold infection by producing various defense-related proteins. Antifreeze activity may be associated with a limited portion of such defense-related proteins.

Disease Resistance Related to Physiological Adaptation

Winter cereals and grasses accumulate fructans, fructose-based polysaccharides, as a storage carbohydrate during cold acclimation. Fructans are utilized as energy source during the winter and plays an important role in both freezing tolerance and snow mold resistance [10]. A comparative study among wheat cultivars with different snow mold resistance indicated that resistant cultivars tend to accumulate higher levels of fructans and consume them more slowly than less tolerant cultivars [45]. Under the deep snow cover where no photosynthesis occurs, fructans are utilized as an energy source to maintain vitality of plants. However, snow molds utilize mono- and disac-

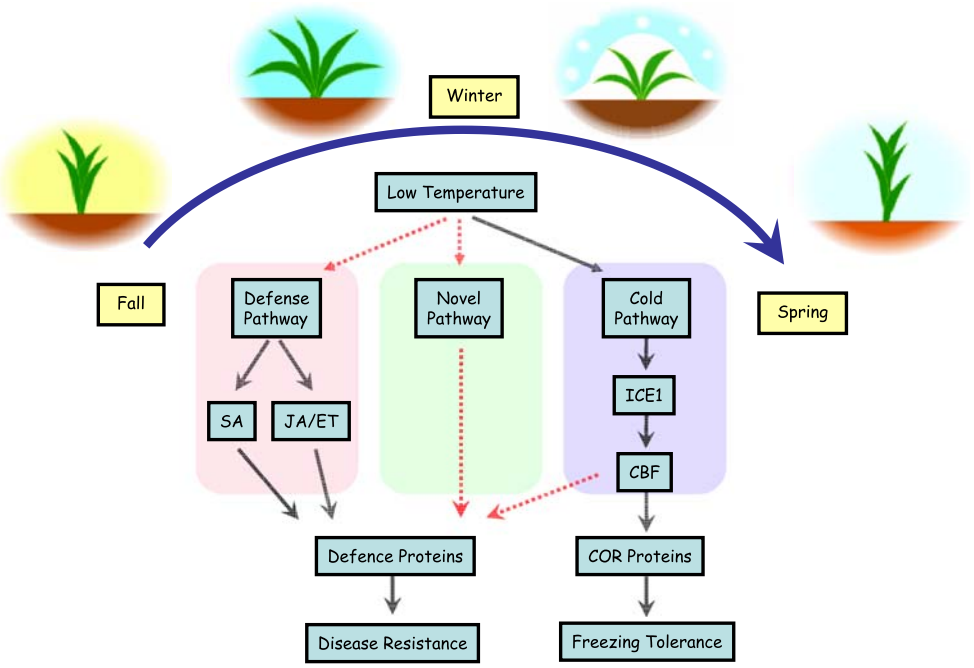

Fig. 4 Model of signaling network for cold-induced disease resistance. *Dashed arrows* indicate unknown pathway

charides derived from fructan degradation in plants. Consequently, such oligo-saccharides promote snow mold infection. Another physiological factor that determines snow mold resistance is cellular dehydration. Dehydration enhances freezing tolerance of plant tissues and is considered one of the major processes of cold acclimation. Since a decrease in water potential significantly inhibits growth of snow molds, dehydration occurs in plant tissues during cold acclimation may contribute to snow mold resistance [35].

Signal Transduction Pathway for Cold-induced Disease Resistance

Although disease resistance and freezing tolerance acquired through cold acclimation are both triggered by recognition of low temperature, it is not known if these two traits are regulated by the same signal transduction pathway. Currently, most extensively studied cold signaling pathway is the CBF pathway. In this pathway, low-temperature-induced CBF transcription factors activate expression of a battery of cold-regulated (COR) proteins that are involved in increasing freezing tolerance (Fig. 4). Genes that are under the CBF regulation have been identified in *Arabidopsis* using microarray [5]. However, no major defense-related genes were classified in this category. In wheat, disease resistance is markedly increased 1 week after onset of cold treatment and then further increased but much slowly (Fig. 2). However, freezing tolerance is acquired incrementally (Fig. 2) [28]. Induction of disease resistance requires light condition, but acquisition of freezing tolerance does not require light [28]. These studies suggested that tolerance against freezing and disease are acquired through distinctive pathways. In addition, while freezing tolerance is readily lost when returned to ambient temperature after cold acclimation, disease resistance partially remains after the transfer [34]. Genetically, these two traits breed independently and cultivars with strong freezing tolerance are generally less resistant to snow molds and vice versa. Cold-induced freezing tolerance and disease resistance may be controlled by independent signaling pathways.

Phytohormones are involved in induction of disease resistance upon pathogen infection. Salicylic acid (SA) and ethylene (ET)/jasmonic acid (JA) pathways play crucial roles in the signal transduction of the disease resistance. It is not known if these pathways are involved in the cold-induced disease resistance. Yu et al. [47] found that antifreeze activity of winter rye was induced by treatments with ET, ethephon (ET-releasing agent), and 1-amino-cyclopropane-1-carboxylate (ET precursor) without cold acclimation while it was not induced by SA and abscisic acid (ABA). Production of endogenous ET reached a peak within 12 h of cold treatment and the level was sustained throughout 1-week cold treatment. It was therefore suggested that ET is a regulator of induction of antifreeze activity in the apoplast of winter rye [46, 47]. On the other hand, apoplastic extracts collected from snow mold-infected winter rye leaves lacked antifreeze activity [13]. These data suggested that ET plays distinctive roles in signaling pathways for cold and pathogen infection. A cold-inducible defensin gene from winter wheat, *TAD1*, was not inducible by either SA or JA treatments, suggesting that TAD1 is a cold-specific isoform of plant defensin family [21]. Together, available data suggested that cold acclimation and pathogen infection activate independent signals and induce distinctive isoforms of antifungal proteins.

Conclusion

Overwintering plants are exposed to continuous cold and snow-covered conditions. In order to endure such environment, plants limit their growth and metabolic activities. This state is considered to be a sort of hibernation. Under such condition, it can be speculated that plants are unable to make quick defense response against pathogen attack. It will be necessary to develop substantial resistance during cold acclimation in late autumn. This is a preventive defense mechanism for plants. Currently, most researches are focused on defense response induced by pathogen, and few studies deal with defense response induced by abiotic cues. It is therefore interesting to dissect signaling pathways from the recognition of cold temperature to the induction of antifungal proteins. Cold-induced defense mechanisms have been studied in winter cereals and grasses. Novel molecular approaches utilizing the model overwintering plant, *Arabidopsis thaliana*, may address a whole sketch of the mechanisms.

Acknowledgments We wish to acknowledge Dr. Yoshiyuki Nishimiya (AIST) for measuring antifreeze activity and Dr. Norio Iriki (NARO) for providing photo. We also acknowledge the input from Drs. Midori Yoshida (NARO) and Tamotsu Hoshino (AIST). Our research on wheat antifungal proteins was supported by a grant from NARO (Development of innovative crops through the molecular analysis of useful genes #3202) to R.I.

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