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Freeze Tolerance Enhanced by Antifreeze Protein in Plant

HWANG, Cheol Ho* · **PARK, Hyun Woo** · **MIN, Sung Ran[1]** · **LIU, Jang Ryol[1]**

Dankook University, Cheonan, 330-714, Korea

[1]Korea Research Institute of Bioscience & Biotechnology, Yusong, 305-333, Korea

ABSTRACT When plants are exposed to subfreezing temperatures ice crystals are forming within extracelluar space in leaves. The growth of ice crystal is closely related to the degree of freezing injury. It was shown that an antifreeze protein binds to an ice nucleator through hydrogen bonds to prevent growth of ice crystal and also reduces freezing damage. The antifreeze proteins in plants are similar to PR proteins but only the PR proteins induced upon cold acclimation were shown to have dual functions in antifreezing as well as antifungal activities. Three of the genes encoded for CLP, GLP, and TLP were isolated from barley and Kentucky bluegrass based on amino acid sequence revealed after purification and low temperature-inducibility as shown in analysis of the protein. The deduced amino acid of the genes cloned showed a signal for secretion into extracellular space where the antifreezing acitivity supposed to work. The western analysis using the antisera raised against the antifreeze proteins showed a positive correlation between the amount of the protein and the level of freeze tolerance among different cultivars of barely. Besides it was revealed that TLP is responsible for a freeze tolerance induced by a treatment of trinexapac ethyl in Kentucky bluegrass. Analysis of an overwintering wild rice, *Oryza rufipogon* also showed that an acquisition of freeze tolerance relied on accumulation of the protein similar to CLP. The more direct evidence for the role of CLP in freeze tolerance was made with the analysis of the transgenic tobacco showing extracellular accumulation of CLP and enhanced freeze tolerance measured by amount of ion leakage and rate of photosynthetic electron transport upon freezing. These antifreeze proteins genes will be good candidates for transformation into crops such as lettuce and strawberry to develop into the new crops capable of freeze-storage and such as rose and grape to enhance a freeze tolerance for a safe survival during winter.

Key words: Antifreeze protein, antifreezing activity, freeze tolerance, transgenic tobacco

Introduction

In plants a cold acclimation was also shown to increase freeze tolerance such that the cold-acclimated rye plants were able to survive at the condition of -30°C otherwise frozen to death at -5°C (Thomashow 1999). There were many biochemical and physiological changes occurred during cold acclimation in the amount of free carbohydrates and proline, the composition of fatty acids in lipid membrane and proteins localized both inside and outside of the cell (Guy et al. 1987; Steponkukus & Uemura 1994; Hwang 1999a). Antifreeze protein is one of the factors increased upon cold acclimation and is known to inhibit a growth of ice crystal by adhering on surface of ice nucleator. The antifreeze proteins were first found in winter flounder and classified to three types of glycoproteins of alanine-rich α-helical, cycteine-rich β-sheet, and no particular structure (Davies & Hew 1990). The antifreeze protein in plants had been reported in the overwintering crops including rye and barley and they were highly homologous to the PR proteins and showed an antifungal activity as well as an antifreeze activity (Hwang 1995; Griffith et al. 1995). However only the PR proteins induced at low temperatures showed antifreezing activity (Mervi et al. 1999). It appears that a formation of

*Corresponding author. Tel 041-550-3626
Email: sfeho@anseo.dankook.ac.kr

complexes with different types of antifreeze proteins, CLP, GLP, and TLP, increases the size and surface to interact with ice crystal and to inhibit growth of ice (Yu & Griffith 1999). Besides, there was a report of isolation and analysis of antifreeze proteins from carrot but they turned out to be different from PR (Smallwood et al. 1999). At this moment only the in vitro assay of the antifreeze proteins purified from rye had shown to possess antifreeze activity but no proof for the activity was shown *in planta*. In this report the cloning and transformation of the gene for antifreeze proteins had been performed in an attempt to show the validity of antifreeze protein in enhancement of freeze tolerance.

Materials and Methods

Plant materials

Barely(Dongbori 1 Ho) was seeded and grown to three leaf stage at 25°C and treated at 4/6°C (day/night: 10/14 hours) for cold acclimation and Kentucky bluegrass (*Poa pratensis L.* cv. monopoy) was cultivated at 25°C under 14 hours light condition. A treatment for cold acclimation was performed at 4/6°C (day/night: 10/14 hours). A 0.02% of trinexapac-ethyl was sprayed onto the leaf as recommended by provider (Young-Il) two times in two consecutive days.

Extraction and analysis of the extracellular proteins

Extracellular proteins from leaf of the barley and Kentucky bluegrass were isolated according to the method described by Hwang (1993). The extracted proteins were concentrated by precipitating with 4 times volume of acetone added at -20°C for 16 hours. A 15% SDS-PAGE was performed to separate the protein and a visualization was done by Coomassie staining. For the western analysis, the antisera raised against the barley TLP purified from the extracellular proteins and CLP purified after being expressed in *E. coli* were used.

Estimation of plant tissue for an antifreeze activity

The leaf blades after removal of tip and base from 10 plants (barely and Kentucky bluegrass) were sliced into 0.5 cm long, and tobacco leaf disk of 0.5 cm diameter were punched. The exudate from cutting edges of the leaf fragment was washed with distilled water and the fragments were lightly dried by blotting with paper towel. Test tubes with a total 0.2 g of the leaf fragments was placed into a low temperature methanol bath stabilized at -2°C. Ice chips were added to initiate freezing and then the temperature of the bath was lowered by 1°C at every 30 minutes. At temperatures of 2°C, -6°C, -10°C, and -14°C each tube was took out and further incubated on ice for 2 hours and then at room temperature overnight before adding 5 ml of distilled water and shaking at 220 rpm for 3 hours at room temperature. The cellular leakage was filtered and then the absorptions at 265 nm were measured using UV-spectrophotometer (Sulc et al. 1991).

Results and Discussions

Antifreeze proteins, the extracellular PR proteins accumulated during cold acclimation

It has been shown that plants accumulate antifreeze proteins in extracellular space of the leaf during cold acclimation. It appears that the protein increased in the amount during cold acclimation has an essential role in an acquisition of freeze tolerance in overwintering plants (Griffith et al. 1993; Hwang 1995). This accumulation can be achieved by low temperature inducibility of the gene for the antifreeze protein as well as extracellular localization of the proteins after their synthesis. In order to isolate the gene for the antifreeze protein, the extracellualr proteins accumulated in response to low temperature stress from barley and Kentucky bluegrass were analyzed as a function of time course. Both plants known to overwinter in nature were shown to increase the amounts of extracelluar protein gradually during cold acclimation at subfreezing temperatures. The extracelluar proteins showing cold inducible accumulation were purified to be analyzed further. Based on data from N-terminal sequencing of the protein they are homologous to the PR proteins such as chitinase, glucanase, and thaumatin like protein. The same result was already reported in rye and these proteins were revealed to show antifreezing activity as well as antifungal activities (Griffith et al. 1994; Griffith et al. 1995). The advantage of the dual functions inherent to antifreeze proteins

can be explained in the light of the chance for plant to expect and prepare for defense to the second attack by pathogen that is inevitably followed after freezing injury. A analogous observation was done in expression of an acidic chitinase gene from a wild tomato, *Lysopersicon chilense*, since the gene was induced by dehydration as well as pathogen stresses (Tabaeizadeh et al. 1999).

Cloning and analysis of genes for antifreeze Proteins, CLP, TLP, and GLP

Based on the properties of cold inducible expression and extracelluar localization, elucidatd from analysis of antifreeze proteins in plants, the strategy to isolate genes for antifreeze proteins was determined. First, the cDNA library constructed with mRNAs induced upon cold acclimation was screened with a heterologous probe to isolate a gene encoding for chitinase like protein (CLP). Second, the PCR using sets of primers designed based on the sequence information from the proteins purified provided genes for glucanase like protein (GLP) and thaumatin like protein (TLP) out of cDNAs made of cold inducible mRNAs.

The analysis of the amino acid sequences derived from three proteins showed that there is a signal sequence encoded to localize them into the extracellular space. Besides, northern analyses showed a gradual increase of the corresponding tanscripts as in an inducible manner by the low temperature. Taken all together, the genes for CLP, GLP, and TLP corresponded to what the plant antifreeze proteins might expect to be, that is the low temperature inducible PR proteins localized to the extracellular space. The CLP was classified to type II and acidic chitinase without a chitin binding domain and a C-terminal signal sequence for vacuole but with a catalytic domain.

Correlation between syntheis of antifreeze protein and freeze tolerance

The antisera against the CLP and TLP were raised either synthesized in *E. coli* or purified from the apoplastic proteins extracted form the leaf, respectively. Western analyses using the extracellular proteins extracted as increasing the period for cold acclimation showed gradual increases in the amounts of extracellular CLP and TLP accumulated during cold treatment. The third leaf emerging in 3 weeks after cold acclimation of rye showed complete a freeze tolerance indicated a close correlation between accumulation of the antifreeze proteins and development of freeze tolerance (Krol et al. 1984; Griffith and McIntyre 1993).

When the extracellular antifreeze proteins from barley cultivars showing different levels of freeze tolerance were analyzed in western, there was a close correlation found between the degrees of freeze tolerance to quantitative aspect of antifreeze proteins accumulated in extracellular space. This could be a strong evidence shwing an essential role of the antifreeze proteins in freeze tolerance and also provide a possibility to use the antisera as molecular marker for quantitative analysis of freeze tolerance in plants.

Freeze tolerance in wild rice, Oryza rufipogon and antifreeze proteins

Oryza rufipogon, a weedy relative of cultivated rice, known to show freeze tolerance, was analyzed by ion leakage analysis. The wild rice showed an acquired freeze tolerance such as a gradual decrease in the amounts of ion leakage as exposed to cold-acclimation condition longer. The acquisition of freeze tolerance by cold acclimation has been rarely found among cultivated rices and *Oryza rufipogon* turned out to be a good source for improvement of cultivated rices in terms of freeze tolerance. However the molecular mechanism to provide the freeze tolerance in *Oryza rufipogon* is not known yet. In order to see the possibility that the antifreeze proteins could play a certain role in the freeze tolerance in *Oryza rufipogon*, the extracellular proteins were analyzed and found *Oryza rufipogon* accumulated several proteins to some degrees before and after cold acclimation but other rice cultivar did only a few and less. As the period for cold acclimation being longer, the amount and number of the proteins got increased. A major protein of 35 KDa in amount was identified to be a CLP in western analysis. Although the 35 KDa CLP was present both *Oryza rufipogon* and other cultivated rice but there was a significant difference in the amount such that *Oryza rufipogon* accumulated much more and increased the level of the protein as cold acclimated longer. It appears that the antifreeze proteins including CLP may allow *Oryza rufipogon* to develop freeze tolerance in response to low temperature in the same man-

ner that other overwintering plants have shown. In addition, the CLP turned out to be a general factor to provide enhanced freeze tolerance in plants.

Trinexapac ethyl-induced freeze tolerance and antifreeze proteins

Trinexapac-ethyl [4- (cyclopropyl-α-hydroxy-methylene)-3,5-dioxocyclohexane carboxylic acid ethyl ester] is a growth-retardant for plants by inhibiting a key step in biosynthesis of GA. A treatment of trinexapac-ethyl generally induces a reduction in vegetative growth (Johnson 1994). Besides the trinexapac-ethyl was known to enhance the freeze tolerance in annual bluegrass however the mechanism is not known yet (Buelow & Rossi 1995). When an acquisition of freeze tolerance by treatment of trinexacpac-ethyl was estimated by ion leakage analysis, a significant increase in freeze tolerance was observed in 50 days after the treatment of trinexacpac-ethyl. In order to see the possible induction of the synthesis of antifreeze proteins by trinexacpac-ethyl, the apoplastic proteins extracted from Kentucky bluegrass treated with trinexapac-ethyl were analyzed by SDS-PAGE. There were several proteins observed to be accumulated as time passed after the trinexacpac-ethyl treatment. A 16 KDa protein was shown to increase in the amount by trinexacpac-ethyl and turned out to be TLP, an antifreeze protein by western analysis (Hwang 1999a). How does a trinexacpac-ethyl induce synthesis of the antifreeze proteins known to be upregulated by low temperature signal? Since GAs and ABA shares the same isoprenoid pathway for their synthesis, an inhibition of GA biosynthesis may lead to *de novo* synthesis of ABA in plant tissues. In many instances, ABA was known to closely relate to the cold hardiness established during cold acclimation and also its treatment could substitute for a cold acclimation to establish the state of cold hardiness in plant tissue as well as plant itself (Rikin et al. 1979; Chen et al. 1983; Chu and Lee 1992). Therefore it appears that an inhibition of GA biosynthesis by trinexapac-ethyl may lead to an increase in ABA contents and then it may enhance freeze tolerance.

Taken together, it may be possible to speculate that the inhibition of GA synthesis by trinexapac-ethyl may boost up the level of endogenous ABA leading to a freeze tolerance, the same effects resulted from a cold acclimation, by way of the synthesis and accumulation of antifreeze proteins in extracellular space.

Analysis of transgenics with CLP and freeze tolerance

The genes for antifreeze proteins, CLP, GLP, and TLP were constructed into a Ti plasmid-derived vector, pGA748 and the introduced into tobacco. The resulting transgenic plants were confirmed to be transformants by PCR showing the fragments of expected size for the NPTII gene and the transgene. The transgenic plants were analysed further to see expression of the transgene and then localization of the protein in apolplast where the antifreeze proteins are supposed to play their role in antifreezing. Western analysis of the proteins extracted from the extracellular space of the transgenic plants showed the accumulation of CLP in apoplast without cold acclimation. This is the first evidence that the antifreeze protein in plants localized into apoplast once synthesized regardless of a treatment at low temperature and the signal sequence inherent to CLP must be all the requirement for extracellular localization. This means that the transgenic plants with the CLP gene expressed can accumulate the CLP playing the role in freeze tolerance in extracellular space even without a pre-treatment at low temperature to guarantee its localization.

In facts, the transgenic tobaccos showed an enhanced tolerance to freezing compared to nontransgenic tobacco grown at the same condition when the freeze tolerance was measured in terms of ion leakage at sub-freezing temperatures. There were some variations found in degrees of tolerance among the transgenic plants but the differences in freeze tolerance were positively correlated to the amounts of CLP accumulated as shown in western analysis. This may be a good indication that the CLP is responsible to the enhanced freeze tolerance observed in the transgenic tobacco. In addition, when the rates of the electron transport from PSII to plastoquinone in transgenic leaf were measured, the CLP transgenic tobacco showed three times less reduction in the rate at the temperature of -10°C compared to that of nontransgenic. This may mean that the transgenic leaf with CLP accumulated was protected from freezing injury. Although the mechanism of the protection against freezing injury on electron transport is not certain, an antifreezing activity of CLP in apoplast may

keep the plasma membrane intact and further protect a possible damage on chloroplast membrane otherwise being injured.

Prospects

Based on molecular properties as well as physiological activities in antifreezing, the genes for antifreeze proteins were proven to be good candidates for transformation into plants for molecular breeding of freeze tolerance. Three of the genes for CLP, GLP, and TLP were on the way to transform into lettuce and strawberry to develop the new crops capable of freeze-storage and manipulation to accumulate the antifreeze proteins within stem of the plants overwintering in the field, such as rose and grape, will also enhance a freeze tolerance for a safe survival during winter.

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