Korean J. Crop Sci. 44(2) : 176~179(1999)

Molecular Analysis of Freeze-Tolerance Enhanced by Treatment of Trinexapac-Ethyl in Kentucky Bluegrass

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ABSTRACT

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 and also inhibits heading. In addition, the trinexapac-ethyl was known to enhance the freeze-tolerance in annual bluegrass, however, the mechanism is not known yet.

One possible reason for the enhanced freeze-tolerance may be the antifreeze protein known to be accumulated in intercellular space of the leaf during cold acclimation. 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 and the presence of the antifreeze protein was observed. In addition, western analysis showed the identity of the protein induced by both a cold acclimation and a trinexapac-ethyl treatment.

It appears that an enhanced freeze-tolerance of the turf grass by trinexapac-ethyl is due to the synthesis and/or accumulation of the antifreeze protein similar to the enhanced freeze tolerance induced by cold acclimation.

Keywords : trinexapac-ethyl, cold acclimation, Kentucky bluegrass, freeze-tolerance, intercellular, antifreeze protein.

Freeze tolerance in plants is determined by several factors such as biochemical, physiological, and physical states. Among them, an instability of plasma membrane due to low temperature is known to be one of the major physical factors leading to freeze injury in plants (Uemura & Steponkus, 1994). The different degrees of freeze-tolerance in plants may depend on the extents of instability of the membrane determined by the differences in composition of fatty acids, in the membrane bound proteins and in the extracellular proteins (Webb & Steponkus, 1993). Dehydration of the plant cells, known to be a direct mechanism for freeze injury, is induced by growth of ice crystals which withdraw water from the adjacent cells and increase the concentration of intracellular contents. In addition, the growing ice crystal in extracellular space finally breaks the adjacent cell wall and membrane and results in irreversible damage to plant tissue(Pearce, 1988). In order to enhance the freeze tolerance in plants, there have been many methods developed, based on cultivation using various fertilizers and breeding against freezing, but there has been only limited achievement so far. Besides, treatments of ABA or other chemicals (GLK-8903) were shown to induce a transient enhancement of freeze tolerance in *Phaseolus vulgaris* (Li et al., 1994).

Trinexapac-ethyl (Ciba Geygi, Greensboro, USA), an inhibitor of GA biosynthesis, is absorbed mainly in the leaf and translocated, and suppresses vegetative growth and retards heading in turf grasses (Johnson, 1994; Fagerness & Penner, 1998). Through suppressing the internodal growth of seedlings and then reducing the chance of lodging by treatment of trinexapac-ethyl, an increased yield was reported in rice production (Im et al., 1993). Besides, a treatment of annual bluegrass with trinexapac-ethyl was shown to increase freeze tolerance along with the accumulation of nonstructural carbohydrates. It was roughly speculated that an antifreeze protein revealed to be accumulated in the extracellular space in other cereal crops may be involved in such an enhanced freeze tolerance (Buelow & Rossi, 1995). This report will show that a treatment of trinexapac-ethyl to Kentucky bluegrass induced accumulation of the leaf extracellular proteins which were identical to those induced to accumulate by cold acclimation. Therefore it appears that the trinexapacthyl may use a similar or identical signal path to increase freeze tolerance as cold acclimation does.

MATERIALS AND METHODS

Plant materials

Kentucky bluegrass (*Poa pratensis*. cv. monopoy) provided from the turf grass laboratory of Dankook University was cultivated at 25℃ under 14 hours light condition. A cold treatment was performed at 4℃ in a low temperature incubator under the same light condition. A 0.02% of trinexapac-ethyl was sp-

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rayed 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 leaves of the Kentucky bluegrass were isolated according to the method described by Hwang (1995). The extracted proteins were concentrated by precipitating with 4 times volume of acetone added at -20℃ for 16 hours. A 15% SDS-PAGE was performed to separate the protein and a visualization was done by the silver staining method (Laemmli, 1970; Oakley et al., 1980). For the western analysis, the antiserum raised against the barley antifreeze protein of 14 KDa (Hwang, 1995) was used.

Estimation of plant tissue for an antifreeze activity

The leaf blades after removal of tip and base from 10 plants were sliced into 2 cm length and the exudates from cutting edges of the leaf fragment were washed in distilled water and the fragments were lightly dried by blotting with a paper towel. Test tubes with a total 0.2 g of the leaf fragments were placed into a low temperature methanol bath stabilized at -2℃. Ice chips were added to initiate freezing and then the temperature of the bath was lowered by 1℃ every 30 minutes. At temperatures of 2℃, -6℃, -10℃, and -14℃ each tube was taken out and further incubated on ice for 2 hours and then overnight at room temperature 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 a UV-spectrophotometer according to the method by Sulc et al. (1991).

RESULTS AND DISCUSSION

An enhanced freeze tolerance by treatment of trinexapac-ethyl

At 30 days after treatment of trinexapac-ethyl, the effect of the chemical on the freeze tolerance was evident at -6℃ and the above as shown in Figure 1-A, but as the duration after the treatment was increased, the effect was shown to be significant only at heavier freezing conditions such as at -10℃ and below, as shown in Fig. 1-C. Based on these results it may be concluded that the treatment of trinexapac-ethyl positively increases the freeze tolerance in Kentucky bluegrass. However, it is not sure whether such enhanced freeze tolerance may be meaningful or not in field conditions. The effectiveness of the trinexapac-ethyl to protect the plants against freezing stress during overwintering in the field remains to be tested in field experiments. The results in Fig. 1 are consistent with those of Buelow et al. (1995) who showed the improved survival of annual bluegrass with a treatment of trinexapac-ethyl. Based on these results they suggested a possible correlation between the enhanced survival and the accumulated nonstructural carbohydrates, and the possibility of an involvement of antifreeze proteins to explain the enhancement of freeze tolerance without any supporting data.

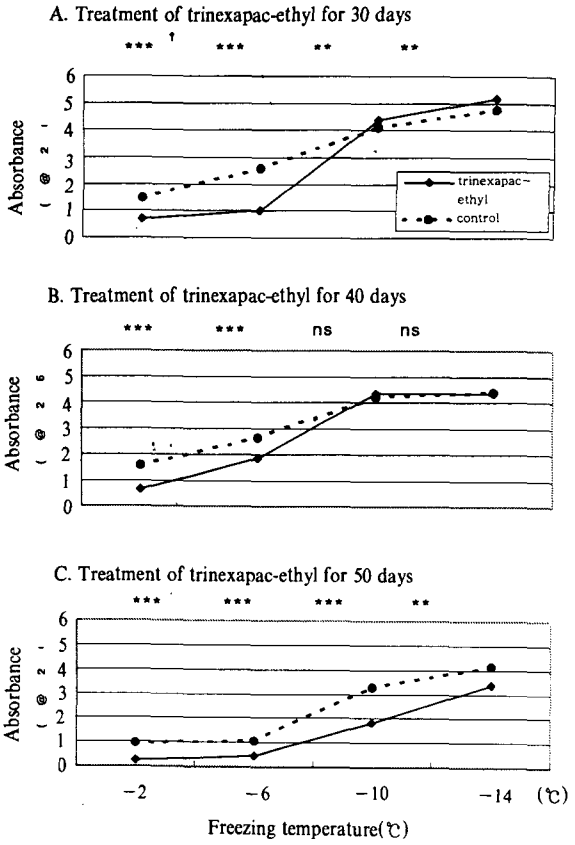

Fig. 1. Spectroscopic measurement of leakage from tissue at freezing temperatures showing that Kentucky bluegrass shows enhanced freeze tolerance at 30, 40 and 50 days after the trinexapac-ethyl treatments. (†; means comparisons between the control and trinexapac-ethyl treatment by t-test, ns: not significant, **, *; significant at 0.01, and 0.001, respectively).**

The extracellular proteins accumulated by treatment of trinexapac-ethyl

Based on the implication of the involvement of antifreeze proteins, induced by treatment of trinexapac-ethyl, in the enhanced freeze tolerance reported

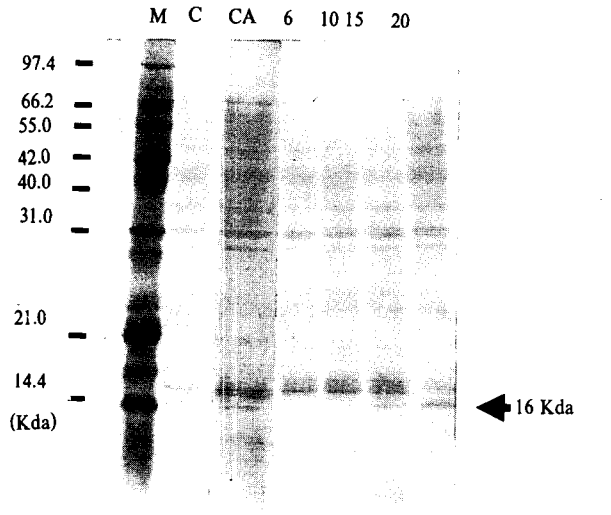

Fig. 2. SDS-PAGE analysis showing accumulation of extracelluar proteins induced by both cold accli- mation and trinexapac-ethyl treatment (M: molecular size markers; C: non-acclimated; CA: cold acclimated; 6, 10, 15, 20: trinexapac-ethyl treated for indicated days).

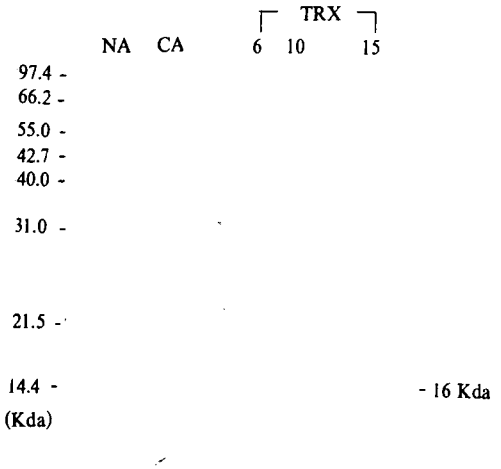

Fig. 3. Western analysis of the apoplastic proteins extracted from Kentucky bluegrass treated with trinexapac-ethyl or cold acclimation using the polyclonal antiserum raised against an antifreeze proteins from barley. (NA :non-acclimated, CA: cold acclimated, TRX: 6, 10, 15 days after treatment of trinexacpac-ethyl).

by Buelow et al. (1995), the extracellular proteins accumulated in Kentucky bluegrass after treatment of trinexapac-ethyl were investigated. The results shown in Fig. 2 indicated that the proteins accumulated in the extracellular space of Kentucky bluegrass in response to both cold acclimation and trinexapc-ethyl treatment were very similar in terms of quantity as well as size. Especially, a 16 KDa protein appears 15 days after trinexapac-ethyl treatment and its amount increased 20 days after the treatment. The 16 KDa protein had been partially sequenced from N-terminal and identified to be the same as an antifreeze protein found in rye (data not shown). Therefore this is the first evidence to support that an enhancement in freeze tolerance by trinexapac-ethyl may be due to the antifreeze proteins accumulated in the extracellular spaces.

In order to see whether the accumulated extracellular protein by trinexapac-ethyl treatment is identical to the antifreeze protein induced by cold acclimation, a western analysis was performed. The result in Fig. 3 indicated that the 16 KDa protein present in the extracellular spaces of the leaf in both the cold acclimated and trinexapac-ethyl treated Kentucky bluegrasses is the same protein, an antifreeze proteins since both proteins were cross-reacted with the antiserum raised against barley antifreeze protein. This may confirm that the enhancement of freeze tolerance by treatment of trinexapac-ethyl is due to an accumulation of antifreeze proteins in a similar or in the same manner, as does the mechanism operated by cold acclimation.

The role of an inhibitor of GA biosynthesis in enhancement of freeze tolerance

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 cold acclimation to establish the state of cold hardiness in plant tissue as well as the plant itself (Rikin et al., 1979; Chen et al., 1983; Chu and Lee, 1992). In addition, there was a report showing a close correlation between the amount of endogenous ABA and the degree of cold hardening (Talyor et al., 1990). 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. In facts, treatments of triazole type growth retardants such as paclobutrazol and flurprimidol also repress biosynthesis of GA, and increase the amount of nonstructural carbohydrates and also establishes a freeze tolerance (Steffen & Wang, 1986; Coleman and Estabooks, 1992; Yim et al., 1997).

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 freeze tolerance, the same effects to be achieved from cold acclimation.

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