Perennial Ryegrass Anti-freeze Protein Genes Enhances Freezing Tolerance in Plants

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Objectives:

1. Measure and compare freezing tolerance of perennial ryegrass cultivars with annual ryegrass cultivars before and after cold acclimation.
2. Characterize functions of the antifreeze protein genes IRIP1 and IRIP2 by overexpressing them in the model species *Arabidopsis* and assess membrane damages and overall survival rate of the transgenic plants under both cold-acclimated and non-acclimated conditions.

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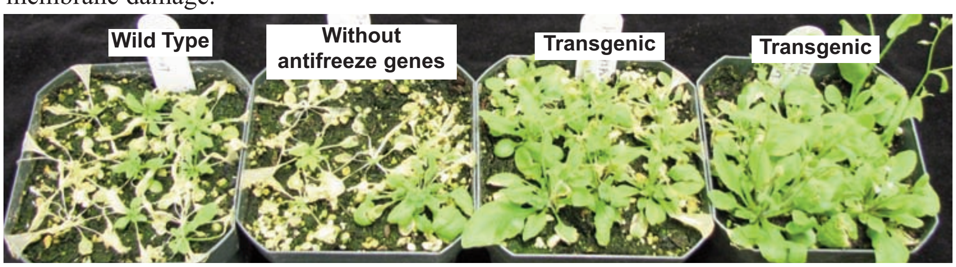
Cold acclimation or cold hardening is an effective mechanism to increase freezing tolerance in temperate plants. Seedlings of two annual ryegrasses cultivars, 'Gulf' (early flowering) and 'Tur' (late flowering), and two perennial ryegrass cultivars, 'Caddyshack' (cold tolerant) and 'Lafayette' (cold sensitive) were used for measuring leakage induced by freezing. Five-week-old seedlings were placed into growth chambers at 23 ºC for two weeks and then acclimated at 4 °C for various periods before they were used for ion leakage test, a measurement of cell membrane damage.



Transgenic Arabidopsis lines ines showed improved survival rates after being subjected to -4, -6, and -8 ºC for 70 minutes compared to a wild type or seedlings carrying the empty vector alone without the antifreeze genes.

Freezing tolerance tests showed that after 14 days of cold acclimation at 4 °C, freezing tolerance greatly increased in both cultivars of perennial ryegrass, but it did not change in the two annual ryegrass cultivars. This suggests that perennial ryegrass can successfully cold acclimate, whereas annual ryegrass cannot. Increasing the length of cold acclimation beyond 14 days, however, did not increase freezing tolerance in perennial ryegrass.

Genes that are highly responsive to cold are detected in higher copy numbers in cold-acclimated perennial ryegrass than in non-cold-acclimated controls. Many genes are highly up-regulated by cold, while some genes are down-regulated. Among the highly upregulated genes, two ice recrystallization inhibition protein (IRIP) genes, LpIRIP1 and LpIRIP2 were identified from a cDNA library constructed from cold-acclimated perennial ryegrass 'Caddyshack'. Reverse transcriptase PCR (RT-PCR) results showed that both genes were highly up-regulated after one day or seven days of acclimation at 4 ºC.

The DNA sequences of both genes suggest that they probably share similar functions with known antifreeze proteins from other plant species. Both LpIRIP1 and LpIRIP2 were transformed into *Arabidopsis* ecotype 'Columbia' through *Agrobacterium*-mediated transformation for overexpression. Transgenic plants are allowed to advance to the T_2 generation. We randomly selected six T_2 transgenic *Arabidopsis* lines carrying LpIRIP1 and another six lines carrying LpIRIP2 gene for freezing and survival test. One-month-old T_2 seedlings were subjected to 70 minutes of freezing temperatures in a programmed freezer. Survival rates were recorded after the plants were recovered in a 22 ºC growth chamber for a week. Without cold-acclimation, two LpIRIP1 transgenic lines (LpIRIP1-7 and LpIRIP1-9) and two LpIRIP2 transgenic lines (LpIRIP2-4 and LpIRIP2-8) showed improved survival rates after being subjected to -4, -6, and -8 ºC for 70 minutes compared to a wild type (Col-0) or seedlings carrying the empty vector alone without the antifreeze genes (EV-6).

The survival rates of the same transgenic lines were also evaluated after cold acclimation. One-month-old seedlings from the selected T_2 lines were acclimated at 4 ºC for eight days before being subjected to freezing temperatures. All seedlings, including wild type (Col-0) and seedlings carrying an emptor vector (EV-6) showed improved survival rates after being subjected to -10, -12, and -14 º C for 70 minutes. The selected LpIRIP1 and LpIRIP2 transgenic lines showed much higher survival rates than exhibited by either the wild type control or the plants carrying the empty vector alone after cold acclimation.

Ion leakage test of these transgenic lines indicated that under non-cold-acclimated condition, only seedlings from LpIRIP1-7 and LpIRIP2-4 showed reduced ion leakage than the control plants Col-0 or EV-6 after freezing at -4 or -5 ºC. However, after cold acclimation, all selected transgenic lines showed reduced ion leakage under either -8, -9 or -10 ºC of freezing comparing to the control plants, although the ion leakage for the control plants also decreased.

Summary Points

- Ion leakage test indicated that perennial ryegrass can successfully cold acclimate, while annual ryegrass is unable to cold acclimate. Two weeks of cold acclimation is sufficient for perennial ryegrass to achieve freezing tolerance, and lengthening the cold acclimation beyond two weeks did not increase freezing tolerance further.
- Overexpressing the two perennial ryegrass antifreeze protein genes, LpIRIP1 and LpIRI P2, in the model species *Arabidopsis* enhanced freezing tolerance as evidenced by the increased survival rate and reduced ion leakage after the transgenic lines were subjected to freezing temperatures.