JARQ 36 (1), 7 – 16 (2002) http://www.jircas.affrc.go.jp

Fundamental Studies on Freezing Hardiness and Wet Tolerance in Italian Ryegrass (*Lolium multiflorum* Lam.) Breeding

Kazuhiro TASE[1]

Department of Lowland Farming, National Agricultural Research Center, Hokuriku Research Center (Joetsu, Niigata 943–0193, Japan)

Abstract

An artificial screening technique based on lethal temperature for 50% kill of plants (LT_{50}) was developed to evaluate the freezing hardiness in Italian ryegrass (*Lolium multiflorum* Lam.) breeding programs. This technique was found to be suitable for evaluating the changes in freezing hardiness in natural and artificial environments, and the freezing hardiness of Italian ryegrass was enhanced through this screening method. To elucidate the mechanisms of freezing hardiness, it was found that the N-terminal amino acid sequence of a protein induced by hardening was similar to the partial amino acid sequence of the abscisic acid (ABA)-inducible protein of alfalfa. ABA thus appeared to be involved in the induction of freezing hardiness in the hardening process. An artificial screening technique based on rooting on the flooded soil surface was also developed to evaluate the wet tolerance. The enhancement of the wet tolerance was achieved through selection using this artificial screening technique. To elucidate the mechanisms of wet tolerance, it was shown that aerenchyma formation in roots under flooding conditions was closely associated with the wet tolerance. Increase in the number of roots with the development of the aerenchyma thus appeared to enhance the wet tolerance. Analysis of the alcohol dehydrogenase (ADH) isozyme and protein synthesis patterns indicated that anaerobic metabolism was possibly induced in roots under flooding conditions.

Discipline: Plant breeding / Grassland
Additional key words: screening technique, amino acid sequence, two-dimensional polyacrylamide gel electrophoresis

Introduction

Since many forage grass species are generally cultivated under various environmental conditions, improvement of resistance to environmental stresses is important for acquiring a stable and high yielding ability. Italian ryegrass is a major winter annual grass in Japan, because of its high palatability for cattle, high yield, etc., and it is widely cultivated in upland fields in rotation with summer crops in the Hokuriku district facing the Japan Sea. However, the wintering ability of Italian ryegrass is lower in terms of snow tolerance and cold hardiness, and in the Hokuriku district, with heavy snowfall and ill-drained fertile heavy clay soils, it often sustains damage due to snow mold and excess water in soil. Improvement of the wintering ability and wet tolerance is thus an important breeding objective for Italian ryegrass. Breeding for snow tolerance has been carried out in the Hokuriku district, and varieties with this ability have been developed. Further improvement of the resistance to other environmental stresses should be promoted for the expansion of the cultivation area and enhancement of the high yielding ability. The present study was conducted for the determination of varietal differences in freezing hardiness and wet tolerance, development of artificial screening techniques, analysis of response mechanisms, and application of artificial screening techniques to Italian ryegrass breeding programs.

Present address:
[1] Yamanashi Prefectural Dairy Experiment Station (Nagasaka, Kitakoma, Yamanashi 408–0021, Japan)
*Corresponding author: fax +81–551–32–3216; e-mail kazu@dairy-exp.pref.yamanashi.jp
Received 22 November 2000; accepted 25 April 2001.

Table 1. Characteristics related to wintering ability of Italian ryegrass

No.	Variety	Origin	Ploidy[a)]	Snow tolerance test (in the field at Joetsu, Niigata)			Cold hardiness test (in the field at Saku, Nagano)		
				Plant survival (%)	Spring growth vigor[c)]	Snow Tolerance Index[d)]	Plant survival (%)	Spring growth vigor[c)]	Cold Hardiness Index[d)]
1	Sakurawase	Japan	2x	19.8 (26.4)[b)]	2.0	11.2	51.4 (45.8)[b)]	3.9	44.2
2	Minamiaoba	Japan	2x	42.4 (40.6)	3.0	25.2	56.7 (48.9)	3.3	39.2
3	Minamiwase	Japan	2x	27.4 (31.5)	2.3	15.2	39.6 (39.0)	3.8	36.0
4	Haruaoba	Japan	2x	66.9 (55.0)	3.5	40.1	58.5 (49.9)	3.3	40.7
5	Waseaoba	Japan	2x	96.9 (79.9)	6.6	109.3	68.6 (56.0)	4.5	61.8
6	Waseyutaka	Japan	2x	70.3 (57.2)	5.1	61.0	57.9 (49.5)	3.5	42.1
7	Niigatakei	Japan	2x	97.1 (80.1)	5.7	95.0	67.4 (57.3)	3.7	52.4
8	Miyukiaoba	Japan	4x	100.0 (90.0)	8.1	150.8	66.6 (54.7)	4.2	56.0
9	Futaharu	Japan	4x	97.4 (81.0)	5.7	96.8	72.6 (58.4)	3.2	46.4
10	Ace	Japan	4x	99.4 (85.6)	6.5	116.6	85.0 (67.6)	5.4	90.2
11	Hitachiaoba	Japan	4x	98.3 (82.6)	6.4	109.2	80.5 (63.8)	4.3	67.9
12	Fujiooba	Japan	4x	99.4 (85.6)	6.7	119.1	80.6 (63.9)	4.6	71.6
13	Gulf	USA	2x	83.0 (65.7)	4.4	59.7	46.4 (42.9)	3.9	40.8
14	Magnolia	USA	2x	36.9 (37.3)	2.6	19.9	55.4 (48.1)	3.7	43.2
15	Imperial	Sweden	2x	72.4 (58.3)	3.9	47.4	74.3 (59.6)	4.3	62.8
16	Tur	Poland	2x	92.7 (74.4)	5.3	81.5	90.2 (71.8)	6.0	105.7
17	Ostsaat-Landsberg	Germany	2x	60.8 (51.3)	3.3	35.4	77.7 (61.8)	3.2	48.7
18	Tetila-Barenza	Netherlands	4x	87.1 (69.1)	5.5	78.8	73.3 (58.9)	4.6	66.5
	Average			74.9 (64.0)	4.8	70.7	66.8 (55.4)	4.1	56.4
	LSD (p=0.05)			4.0	0.4		5.3	0.5	

a): 2x, Diploid; 4x, Tetraploid.
b): Figures in the parentheses show angular transformed values.
c): Spring growth vigor in Saku and Joetsu was visually estimated on Mar. 26, 1991 and Apr. 3, 1991, respectively[1(poor)–9(good)].
d): Index = [(percentage of plant survival) × (spring growth vigor) / (average of spring growth vigor of all varieties used)], calculated by using the data in each field test.

Varietal differences in freezing hardiness and development of screening technique

1) Evaluation of wintering ability in natural environments

The wintering ability of Italian ryegrass varieties was evaluated based on snow tolerance and cold hardiness using 18 varieties from September 1990 to March 1991 in an experimental field located in a heavy snowfall area (National Agricultural Research Center for Hokuriku Region) and a cool highland area (Nagano Station of National Livestock Breeding Center). As selection indicators for the wintering ability, Snow Tolerance and Cold Hardiness Indices were determined, based on the winter survival and spring growth vigor of plants (Table 1). A significant but weak relationship was found between these indices (Fig. 1), i.e. Miyukiaoba showed a higher snow tolerance than cold hardiness, while Tur showed a higher cold hardiness. Ace and Fujiooba were relatively superior in both snow tolerance and cold hardiness. Cold Hardiness Indices for Miyukiaoba and Niigatakei, varieties bred in the Hokuriku district with a high snow tolerance were lower than the average value of all the varieties, suggesting that the varieties selected with a strong snow tolerance may not always show a high cold hardiness.

2) Development and utilization of an artificial screening technique for freezing hardiness

An artificial screening technique was developed based on the method of Fuller and Eagles[2)] for the evaluation of cold hardiness in perennial ryegrass (*L. perenne* L.). Screening of freezing hardiness was conducted as follows (Fig. 2) : Seedlings were raised in a growth cabinet at day / night temperatures of 20 / 15ºC under a 16 h photoperiod of 15,000 lx for 4 weeks. The seedlings at the 3–4 leaf stage were hardened in the growth cabinet at 2ºC under a 8 h photoperiod of 15,000 lx for 14 days.

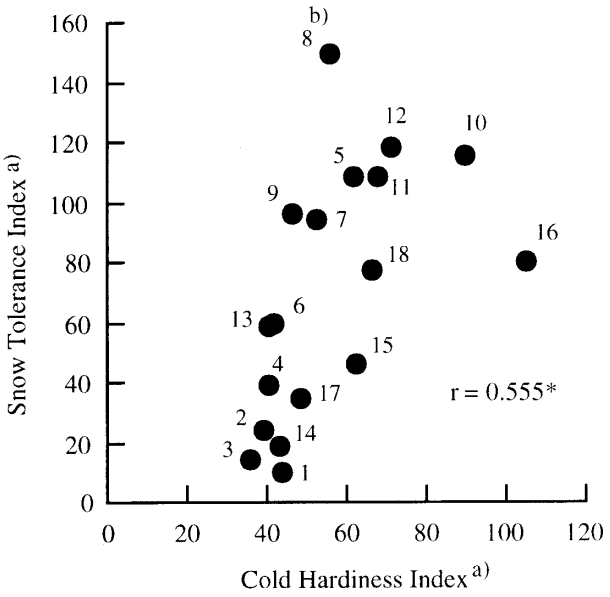

Fig. 1. Relationship between Snow Tolerance Index and Cold Hardiness Index

a): See the footnote d) of Table 1.
b):Variety numbers are the same as those in Table 1.
*Significant at 5% level.

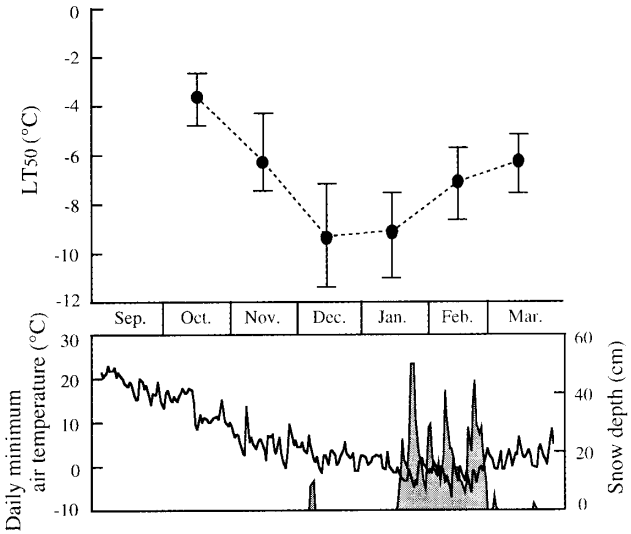

Fig. 3. Changes in average LT50 value of 18 Italian ryegrass varieties, daily minimum air temperature and snow depth during the 1991–92 winter at Hokuriku Research Center

⊥ : Range (minimum and maximum of LT50 of varieties used),
▨ : Snow depth,
— : Daily minimum air temperature.

Following hardening, they were subjected to freezing treatments in which the temperature was lowered to the fixed temperature of –4ºC to –12ºC at 2ºC intervals at a cooling rate of –1ºC / h, followed by 16 h plateaus at the designed temperature, respectively, thawed at a heating rate of –2ºC / h, and kept at 2ºC for 24 h thereafter. The seedlings were then maintained for 3 weeks under ordinary growing conditions. Plant mortality was recorded and the LT50 value was determined by probit analysis to evaluate the freezing hardiness. The artificial screening technique was applied to examine the changes in freezing hardiness during winter in 18 Italian ryegrass varieties. Plants were sampled from the field of the National Agricultural Research Center for Hokuriku Region once a month from October until March. Degree of freezing hardiness based on LT50 values increased rapidly until December and then decreased gradually. This pattern appeared to be closely related to the daily minimum air temperature (Fig. 3).

Effects of hardening on the development of freezing hardiness

Many plant species develop freezing hardiness when exposed to low non-freezing temperatures, a process generally referred to as hardening[3]. Eleven varieties were

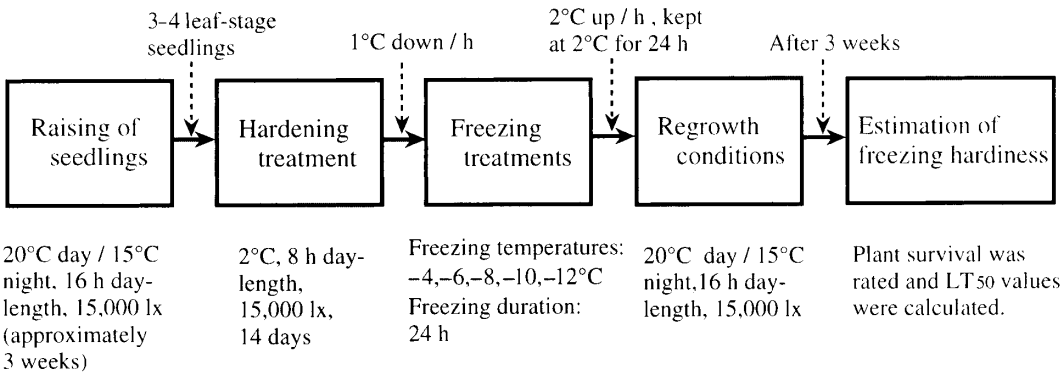

Fig. 2. Scheme of artificial screening technique for freezing hardiness in Italian ryegrass

sown 4 times at 2-week intervals from September 14 to October 26 in natural environments. Freezing treatment was applied at the 4–5 leaf stage of the seedlings. The effects of hardening, as determined from the changes in the LT50 values, were rapidly induced below a daily mean air temperature of 10ºC after early November.

To investigate the influence of the temperature, photoperiod and duration of the treatment on hardening, Waseaoba (slightly high freezing hardiness) and Minamiwase (low freezing hardiness) were grown and the seedlings were treated basically as shown in Fig. 2. Freezing hardiness of either variety increased with the decrease in the temperature from 10 to 2ºC. The threshold temperature for hardening of Waseaoba was about 10ºC, while for Minamiwase between 6 and 4ºC (Fig. 4). Neither short (8 h) nor long (16 h) photoperiod had a significant effect on either variety at lower temperatures (6 and 2ºC), though at a higher temperature (10ºC), freezing hardiness slightly increased under the short photoperiod. The effects of hardening with declining temperature were thus much more pronounced than those with the change in photoperiod. Hardening was noted at more than 14 days at 2ºC and under a 8 h photoperiod for either variety. These results are considered to provide important information for the growth of plant materials with sufficient hardening in natural or artificial environments.

Mechanism of freezing hardiness

1) Screening of hardening-related proteins

Since *L. multiflorum* shows considerable variations in genotype, it is less suitable for biochemical or molecular biological studies. *L. temulentum* L., a self-pollinated species, shows a highly homogeneous growth. Thus, the hardening-related proteins in *L. temulentum,* strain PI 176624, were studied first[8]. Proteins extracted from non-hardened and hardened seedlings for 14 days were analyzed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). One newly induced protein and 6 others showing quantitative increases were identified in the hardened seedlings but not in the non-hardened ones (Fig. 5). The N-terminal amino acid sequences of these proteins were determined using a gas-phase protein sequencer. A homology search of amino acid sequences with a protein sequence data bank indicated that several proteins showed a high homology with the ABA-inducible protein of alfalfa (*Medicago sativa* L.)[5], ribulose-bisphosphate carboxylase large-subunit binding protein in garden pea (*Pisum sativum* L.) and leaf fructose diphosphate aldolase in spinach (*Spinacia oleracea* L) (Fig. 6). For the other proteins, N-terminal amino acid sequences could not be determined owing to the block at the N-terminal of the proteins.

2)Effects of ABA on the development of freezing hardiness

ABA is considered to play an important role in the hardening process for adaptation to freezing stress. Changes in freezing hardiness and endogenous ABA content with exogenous ABA treatment were studied in relation to those of hardening[9]. Treatment with 7.5×10^{-5} M ABA led to the highest level of freezing hardiness, when ABA was sprayed on leaves at intervals of 12 h at room temperature. ABA treatment increased the freezing hardiness to some extent until 14 days but only slightly during the hardening treatment thereafter (Fig. 7). ABA treatment also led to a more rapid and larger increase in the endogenous ABA content during the treatment, com-

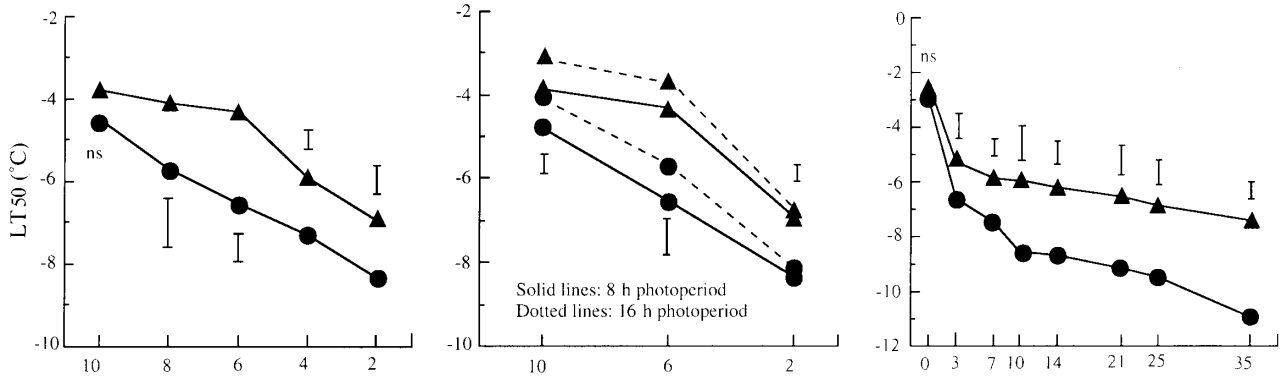

Fig. 4. Effects of temperature, photoperiod and duration on the hardening of 2 Italian ryegrass varieties
● : Waseaoba, ▲ : Minamiwase, ns: Not significant.
Vertical bars indicate LSD at 5% level.

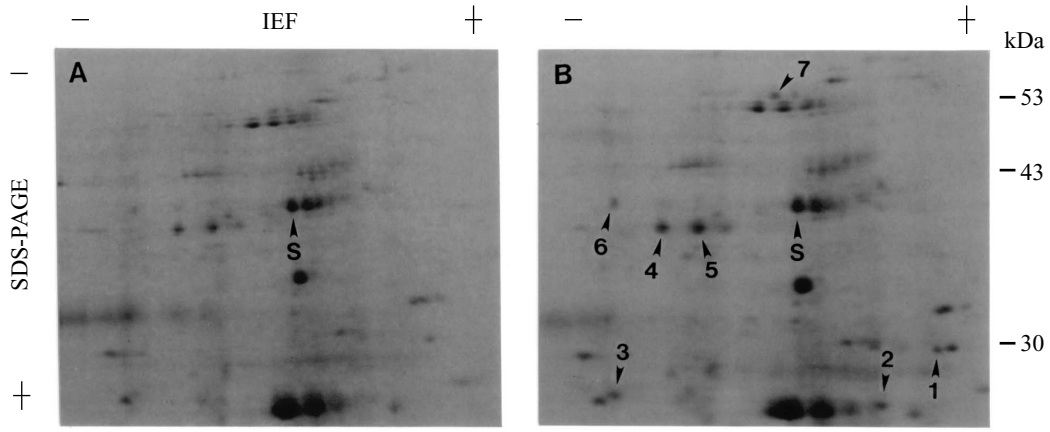

Fig. 5. Changes in pattern of protein synthesis of *L. temulentum* strain PI 176624 with hardening
A: Non-hardened seedlings (control), B: Hardened seedlings (2ºC, 8 h photoperiod for 14 days).
Spot No. 1: Newly induced protein, Spot No. 2-7: Increase in the amount of proteins by hardening,
Spot marked S: Standard spot for measuring the amount of proteins.
Left to right: Isoelectric focusing (IEF) for the first dimension.
Top to bottom: SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for the second dimension.

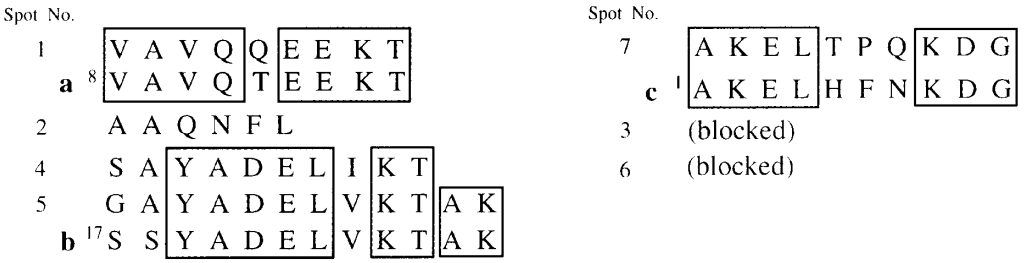

Fig. 6. N-terminal amino acid sequences of proteins induced or with an increased amount by hardening
a: Abscisic acid (ABA)- inducible protein (*Medicago sativa*), b: Leaf fructose diphosphate aldolase (*Spinacia oleracea*), c: Ribulose-bisphosphate carboxylase large-subunit binding protein (*Pisum sativum*).
Identical amino acids of homologous proteins are indicated by solid boxes.
Spot numbers correspond to those shown in Fig. 5.
The numbers indicate the positions of residues in the published sequences.

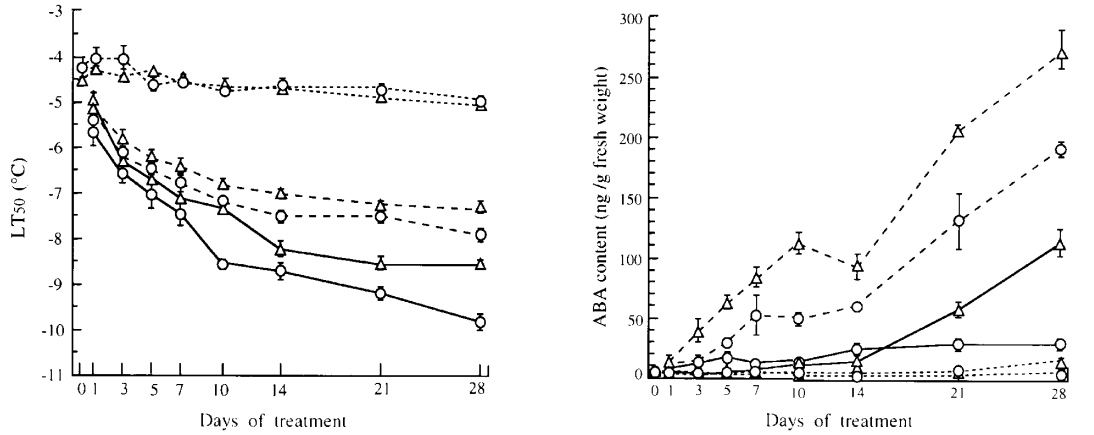

Fig. 7. Changes in freezing hardiness and endogenous ABA content with hardening or exogenous ABA treatment in *L. multiflorum* and *L. temulentum* seedlings
○ : Waseaoba, △ : PI 176624.
——— : Hardening treatment (2ºC, 8 h photoperiod for periods indicated),
– – – : ABA treatment (7.5×10^{-5} M at 20ºC day / 15ºC night, 16 h photoperiod),
- - - - - : Control (20ºC day / 15ºC night, 16 h photoperiod).
Vertical bars represent the standard errors of the mean.

pared with the hardening treatment. Since the accumulation of endogenous ABA was not correlated with the increase in freezing hardiness, ABA might have triggered the hardening. However, freezing hardiness may not depend on endogenous ABA levels only.

Varietal differences in wet tolerance and development of screening technique

1) Evaluation of wet tolerance by principal component analysis

Principal component analysis was applied to identify the major component using data of 9 characteristics of seedlings from 20 varieties and strains after the flooding treatment. The first principal component was considered to be a determinant of wet tolerance (Table 2). A high correlation was recognized between the first principal component score and the dry weight under flooding conditions. This score was thus considered to be useful for the evaluation of wet tolerance[7].

2) Development of an artificial screening technique for wet tolerance

Rooting on the soil surface after flooding, which corresponds to the morphological adaptation to avoid anoxia, was observed[1], and remarkable varietal differences were noted in the degree of rooting (Fig. 8)[7]. The degree of rooting was closely correlated with the relative dry weight (flooded/non-flooded) of roots in the upper layer of soil after flooding ($r = 0.801^{**}$) and also significantly with the first principal component score in flooding ($r = 0.708^{**}$). Examination of the degree of rooting was thus found to be easier than principal component analysis for evaluating wet tolerance. Screening of wet tolerance was conducted as follows: Seedlings were raised in a growth cabinet at day / night temperatures of 20 / 15ºC under a 16 h photoperiod of 15,000 lx for 4 weeks. The seedlings at the 3–4 leaf stage were flooded with water at a 5 cm depth for 2 weeks at a constant temperature of 25ºC. The shoots were removed from the plants to observe clearly the state of rooting on the soil surface. The degree of rooting was evaluated based on the area with roots on the soil surface.

Analysis of the mechanism of wet tolerance

1) Development of aerenchyma in roots after flooding treatments

Aerenchyma formation in roots is considered to be an adaptive response to flooding in higher plants as well as rooting on the soil surface, and it plays an important role in the transport of oxygen from leaves to roots[4]. To examine the relation between wet tolerance and aerenchyma formation, intercellular spaces in root tissues were observed with a microscope (Fig. 9). In Miyukiaoba and Fujiooba which display a high wet tolerance, intercellular spaces expanded under flooding conditions, unlike in Futaharu and PI 311421 which exhibit a low tolerance. Aerenchyma formation is thus likely to be closely associated with wet tolerance. Roots on the soil surface showed an extensive formation of aerenchyma in every variety. Increase of the number of roots with aerenchyma thus appeared to result in a high wet tolerance.

Table 2. Eigen vector of 9 characteristics in the first 2 principal components

Characteristics	Principal components	
	1	2
Plant height	0.588	–0.629
Number of leaves on the main stem	0.726	0.105
Number of tillers	0.660	0.195
Number of green leaves	0.891	–0.101
Fresh weight of shoots	0.881	–0.197
Dry weight of shoots	0.801	0.252
Fresh weight of roots	0.795	0.193
Dry weight of roots	0.665	0.426
Leaf color	0.263	–0.800
Eigenvalue	4.664	1.415
Percentage of eigenvalue	51.8	15.7

Statistical analysis was performed using values calculated by (flooding treatment – control) × 100 / control.

2) Changes in ADH isozyme activity and protein synthesis associated with flooding treatment

It has been considered that flooding conditions preclude aerobic metabolism so that root survival becomes dependent upon metabolic activities such as anaerobic fermentation[6]. ADH and lactate dehydrogenase (LDH), i.e. enzymes related to the fermentative pathway were selected for the study of anaerobic metabolism under anaerobic conditions of flooding. Changes in the ADH isozyme activity of roots under flooding and non-flooding conditions were investigated by native polyacrylamide gel electrophoresis using varieties and strains differing in wet tolerance. The isozyme pattern of ADH extracted from the roots of the seedlings in each treatment was the same among the varieties and strains used, though the ADH activity increased under flooding conditions (Fig. 10). Thus, no distinct relationship was found between wet tolerance and the ADH isozyme pattern[7].

To identify the proteins that may be essential for wet tolerance, root proteins extracted from seedlings in Waseaoba grown under flooding and non-flooding condi-

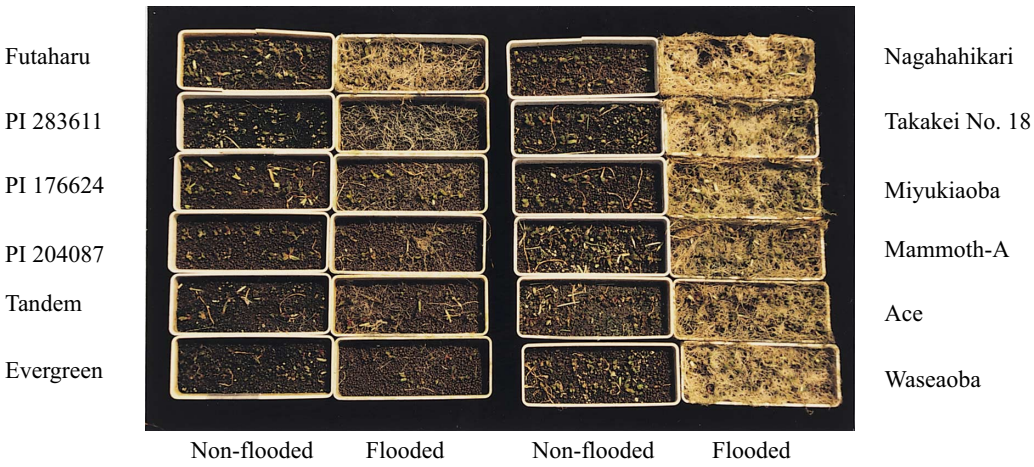

Fig. 8. Varietal defferences in the degree of rooting on soil surface with flooding treatment for 2 weeks
The shoots were removed from the plants to observe clearly the state of rooting after the flooding treatment.

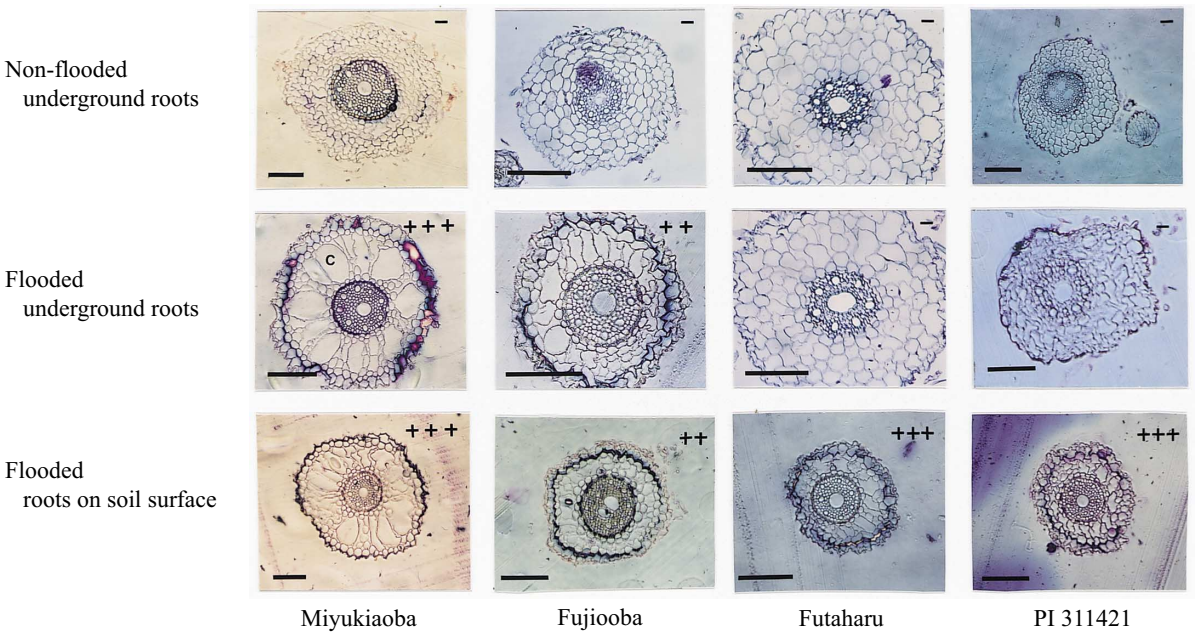

Fig. 9. Typical transverse sections of the basal portion of roots in non-flooded and flooded seedlings
Sections were prepared from the basal 2.5–3.0 cm sections of roots. c: Cortical air space (aerenchyma). Bars represent 20 µm. Miyukiaoba, Fujiooba: Varieties with high wet tolerance. Futaharu, PI 311421: Variety and strain with low wet tolerance. Symbols (–: null, +: low, ++: moderate, +++: high) on the upper right within figures indicate the degree of development of the cortical air space.

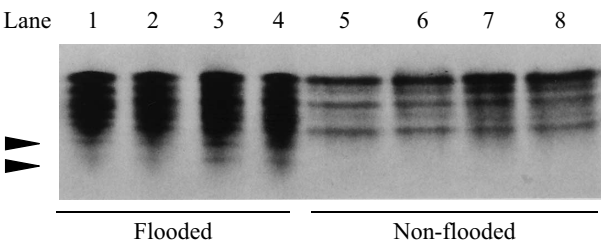

Fig. 10. Isozyme patterns of alcohol dehydrogenase (ADH) in roots of flooded and non-flooded seedlings
The soluble proteins were extracted from the roots of seedlings subjected to flooding or under non-flooding conditions for 10 days. Isozymes were separated by native polyacrylamide gels. The gels were stained for ADH activity. Arrowhead indicates bands observed only in flooded seedlings. Lanes 1 and 5: Futaharu, lanes 2 and 6: PI 311421, lanes 3 and 7: Tetrelite, lanes 4 and 8: Miyukiaoba.

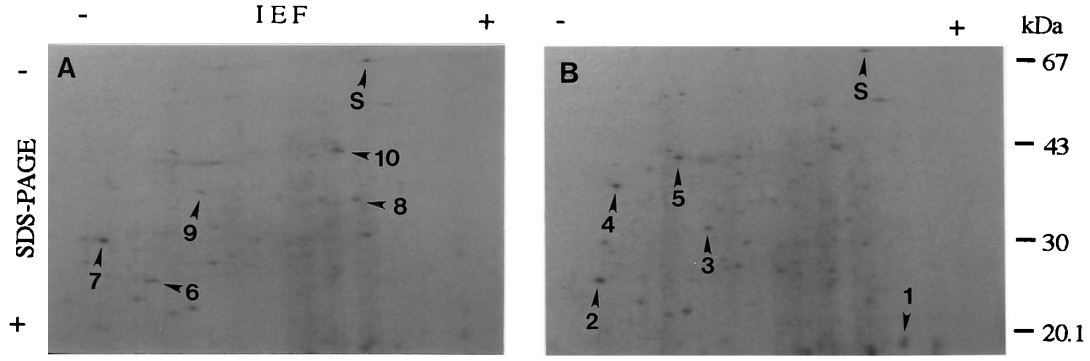

Fig. 11. Changes in the pattern of protein synthesis in roots of *L. multiflorum* variety Waseaoba with flooding treatment
A: Non-flooded seedlings, B: Seedlings flooded for 10 days.
Spot No.1, 3–5: Increase of protein amount in flooded seedlings, Spot No.2: Newly induced protein,
Spot No. 6–10: Decrease of protein amount in flooded seedlings.
Spot marked S: Standard spot for measuring the amounts of proteins.
Left to right: IEF for the first dimension, Top to bottom: SDS-PAGE for the second dimension.

Spot No.			
1			E D A G N N G D I E G
	a	57	V R A G S Y G D L E G
7			G E T S D V G G N I A D A G
	b	248	G E I C D I N G N C V D A A
8			T D A E E N G E E G Q E A
10			Q T A Q T I G E I G D L E Q G
2-6,9			(blocked)

Fig. 12. N-terminal amino acid sequences of proteins induced and with increased or decreased amount by flooding treatment in *L. multiflorum* variety Waseaoba
a: L-lactate dehydrogenase (*Thermus aquaticus*),
b: H^+-transporting ATP synthase γ chain precursor (*Pisum sativum*).
Identical amino acids of homologous proteins were indicated by solid boxes.
Spot numbers correspond to those shown in Fig. 11.
The numbers indicate the positions of residues in the individual published sequences.

tions were analyzed by 2D-PAGE. One newly synthesized protein and several proteins with quantitative variations were identified in the flooded seedlings (Fig. 11). The N-terminal amino acid sequences were determined in the same manner as that for the analysis of hardening-related proteins, and a homology search was also conducted. Protein with a quantitative increase was homologous to LDH which belongs to fermentative enzymes (Fig. 12). For the other proteins, N-terminal amino acid sequence determination was not possible owing to the block at the N-terminal of proteins.

Application of artificial screening techniques to the breeding programs

To enhance the freezing hardiness of Miyukiaoba and Waseaoba, both of which display a high snow tolerance, plants selected from each variety through screening at a single freezing temperature of –9ºC were crossed with each other. This selection of surviving plants was repeated for 2 generations. LT_{50} value of each generation was clearly lower than that of each parent variety (Table 3). Freezing hardiness in Italian ryegrass thus could be

Table 3. Effects of selection for freezing hardiness in Italian ryegrass

Original variety	Generation in selection	Selection method	No. of plants raised	No. of selected plants	Selection rate (%)	LT_{50} (ºC)
Waseaoba	Origin					–8.4a
	1st generation	Mass selection	972	148	15.2	–9.0b
	2nd generation	Mass selection	972	397	40.8	–9.3b
Miyukiaoba	Origin					–7.4a
	1st generation	Mass selection	972	57	5.9	–7.9b
	2nd generation	Mass selection	972	121	12.4	–8.3c

Freezing temperature was –9ºC.
LT_{50} values with different letters within the same variety group are significantly different ($P<0.05$).

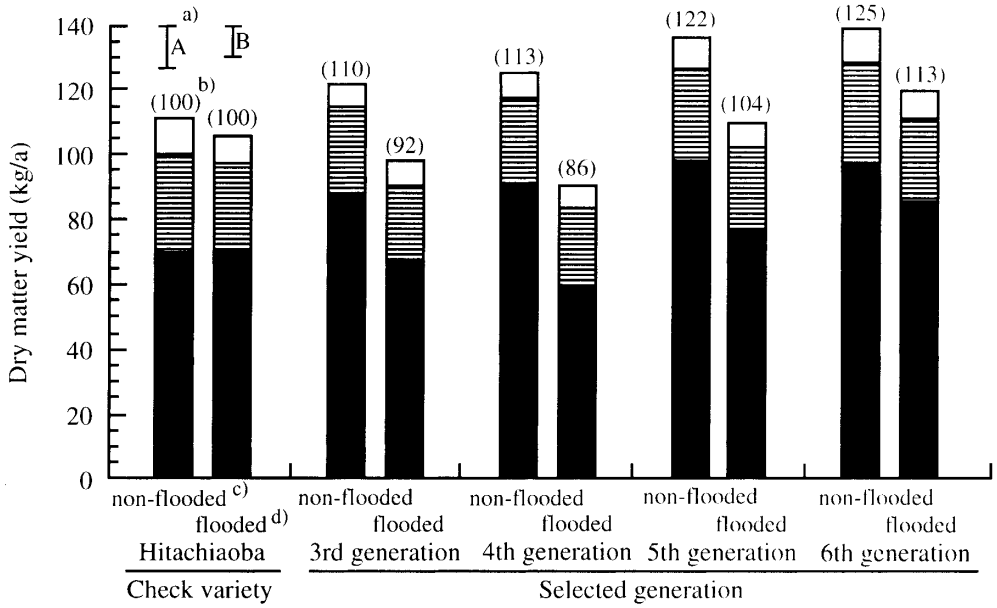

FIg. 13. Dry matter yield of each selected generation after flooding and without flooding treatment in the field

: 1st cutting, : 2nd cutting, : 3rd cutting.

a): Vertical bars (A and B) indicate LSD at 5% level without flooding and with flooding treatment, respectively.

b): Figures in the parentheses show the percentage of dry matter yield to the check variety Hitachiaoba in each treatment.

c): Plants were grown under normal conditions (left).

d): Plants were flooded by watering up to 7 cm from the soil surface, from the 4–5 leaf stage until just before the 1st cutting, except for the winter duration (right).

improved through selection by artificial screening. In addition to the ordinary mass selection and maternal line selection methods applied for various agricultural characteristics, the selection for wet tolerance was performed for several generations through screening based on the degree of rooting after flooding. Owing to the high degree of rooting, wet tolerance was remarkable and dry matter yield in subsequent selected generations exceeded that of the check variety (Fig. 13).

The screening techniques proposed in this study may enable to accumulate the potential capacity for freezing hardiness and wet tolerance in Italian ryegrass, and should be useful for improving the resistance to environmental stresses.

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