CHARACTERIZATION OF SOMATIC HYBRIDS BETWEEN FESTUCA ARUNDINACEA AND LOLIUM MULTIFLORUM

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ABSTRACT
Metabolically inactivated tall fescue (*Festuca arundinacea* Schreb.) protoplasts and X-ray irradiated Italian ryegrass (*Lolium multiflorum* Lam.) protoplasts were electrofused resulting in the regeneration of twenty-six plantlets. Southern blot analysis using three mitochondrial probes and random amplified polymorphic DNA (RAPD) analysis revealed that the regenerants were intergeneric somatic hybrids. All regenerated plants were male sterile and some of them had stamens that exhibited the pistillode appearance.

INTRODUCTION
Tall fescue is an important grass species widely used for agricultural and recreational purposes in Japan. However, because of its wide adaptation it has also become a social problem due to its production of allergens that induce pollinosis in human beings. It is our implicit assumption that this problem could be taken care of adequately if phenotypes with nonfunctional anthers, such as cytoplasmic male sterile lines are developed using the tools of genetic engineering.

The study was designed to develop cytoplasmic male sterile tall fescue lines through transfer of the cytoplasmic male sterility (CMS) trait from Italian ryegress using asymmetric protoplast fusion.

MATERIALS AND METHODS
Isolation, fusion and culture of protoplasts. Hexaploid (2n=42) tall fescue cultivars Nanryo, Manade and Yamanami, and diploid (2n=14) Italian ryegrass cv. Waseaoba, earlier identified by Komatsu (1987) to possess the cytoplasmic male sterile trait were used for the establishment of suspension and protoplast cultures as described previously (Takamizo et al., 1990).

Italian ryegrass protoplasts were irradiated at 80 and 160krad with X-ray apparatus (OM-100R, Ohmic, Japan) operated at 80KV, 8mA, 15cm. Tall fescue protoplasts were treated with 10mM iodoacetamide in liquid CPW13M (that is, CPW medium containing 0.7M mannitol) for 15 min at room temperature. Treated protoplasts were washed twice by centrifugation in CPW13M. Fusion experiments were performed with a commercial electrofusion apparatus (Shimadzu SSH-1, Shimadzu, Japan) using the following conditions: AC-field (0.5MHz, 30V for 20-30s) followed by DC-pulses (20µ-s, 1.25kV/cm, 1-2 pulses).

Plant regeneration. Proliferating callus pieces after about a month in culture were transferred onto MSSSD1P as described by Takamizo et al. (1990).

Southern hybridization and RAPD analyses. DNA was isolated from young leaves of both tall fescue and Italian ryegrass as well as the asymmetric somatic hybrid plants derived from them using the CTAB method. The DNA was digested with EcoR1, electrophoresed in 0.8% agarose gel and transferred to nylon membranes. Reagents and protocols for labelling, hybridization and detecting DNA were adapted from ECL system of Amersham. PCR reactions were carried out in 10µ-l volumes containing 10mM Tri-HCl (pH8.3), 50mM KCl, 1.5mM $MgCl_2$, 400µ-l dATP, dTTP, dCTP, dGTP, 0.75U TaKaRa Taq polymerase,100ng genomic DNA and random primers in TaKaRa PCR Thermal Cycler under the following conditions: denaturation temperature (94°C; 1min), annealing temperature (45°C; 2min) and extension temperature (72°C; 3min) for 35 cycles.

RESULTS AND DISCUSSION
Plant regeneration. A test of four different genotypic fusion combinations resulting in the regeneration of twenty-six asymmetric plantlets expected that the regenerants contained a complete nucleus derived from tall fescue, an incomplete Italian ryegrass nucleus and a mixture of the cytoplasms from both grass species. All regenerated plants were morphologically identical to tall fescue. For example, the leaves and inflorescences of the regenerants had a similarity with those of tall fescue.

Analyses using Southern hybridization and RAPD. Five regenerated plants used for Southern blot analysis with three mitochondrial gene-specific probes *cox1*, *cox2* and *atp6* revealed that all tested plants were somatic hybrids.

Sixteen regenerated plants used for random amplified polymorphic DNA (RAPD) analysis. indicated that all regenerants had tall fescue specific bands. Moreover, some of these also possessed Italian ryegrass specific bands; an indication of the presence of Italian ryegrass chromosomes in the regenerants. It could be speculated from these observations that the dosage of X-ray used for irradiation was perhaps too low to enhance complete elimination of the Italian ryegrass genome.

Pistillody of stamens
It is indicated by our data in Table 1 that of the twelve regenerants showing complete male sterility, eight also exhibited pistillody of stamens while four did not. It is possible that the latter were all derived from a single experiment.

Pistillody was observed when the CMS cytoplasm of Italian ryegrass was transferred to tall fescue by backcossing. Italian ryegrass (designated here with an LL genome composition) with CMS cytoplasm when crossed with tall fescue (designated with an AABBCC genome) resulted in the production of hybrids whose F1 seeds carried the ABCL genome. Amphidiploids (with AABBCCLL genome) were obtained when treated with colchicine. In the next generation (BC1=backcross) when backcrossed with tall fescue, some plants exhibited pistillody. However, F1 hybrids and amphidiploids did not show pistillody of stamens. In both cases pistillody was not completely exhibited in the genome of Italian ryegrass; presumably an indication that pistillody is incompletely exhibited in Italian ryegrass plants having genome with CMS cytoplasm.

Collectively, it could be concluded from these observations that: 1) Plants derived from asymmetric cell fusion were obtained, 2) these plants had a part of the CMS cytoplasm and genome of Italian ryegrass, and 3) all regenerated plants were male sterile and some of them also exhibited pistillody.

REFERENCES
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Table 1
Conditions of electrofusion and frequency of regenerated plants showing pistillody

Combination of cell fusion			Number of	Frequency of
TFa	IRb	X-ray dose	plants regenerated	Pistillody
YA2	MS17	80[c]	4	0
MA2	MS17	80	4	100
NA13	MSpro	160	3	100
MA2	MS17	200	1	100

[a] Tall fescue varieties: YA2(Yamanami), MA(Manade) and NA(Nanryo)
[b] MS17 and MSpro were cell suspensions. MS17 was derived from Italian ryegrass clone possessing CMS cytoplasm and Mspro was derived from progeny seeds of MS17.
[c] krad

Figure 1
Floral organs of asymmetric somatic hybrids between tall fescue and Italian ryegrass. Note that in (A) stamens had developed the pistillode appearance, whereas in (B) stamens maintained their normal appearance. Both types, however, were completely male sterile.