Screening of Regenerable Genotypes of Italian Ryegrass
(Lolium multiflorum Lam.)

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Abstract: We screened regenerable genotypes from 12 cultivars of Italian ryegrass (Lolium multiflorum Lam.) through tissue culture of mature seeds, shoot tips and root tips. Although three morphological types of calli, friable, watery, and compact with fine root hair were observed, green shoots regenerated only from the friable calli. The highest frequency of the formation of regenerable callus from mature seeds was 1.4% in the diploid cultivar Waseaoba and 1.1% in the tetraploid cultivar Meritra. The plants regenerated from mature seed-derived calli were maintained and propagated aseptically in vitro, and the method of regenerating plantlets via callus culture was established by using their shoot tips. Sixty-three seeds of Waseaoba and 44 seeds of Meritra were aseptically sown, each seedling (genotype) was propagated in vitro, and the shoot tip from each genotype was subjected to tissue culture. More than 22% of these genotypes formed regenerable calli. From these results, we conclude that the shoot tip of the in vitro-preserved plantlet is useful for producing the regenerable calli routinely. The information obtained in the present study is useful for establishing a reliable transformation system for Italian ryegrass.

Key words: Plant regeneration, Tissue culture.

Italian ryegrass (Lolium multiflorum Lam.) originated in the Mediterranean region and is one of the most important forage grasses in the temperate region. It was introduced to Japan in the early Meiji era (1870s) and is now cultivated in a wide area from Hokkaido to Kyusyu. Because it is an outcrossing crop and is thus self-inferile, the breeding of this crop using selection proceeds slowly.

Recently, biotechnological approaches including somatic hybridization and genetic engineering are being applied to crop breeding. By using these approaches, agronomically useful genes can be introduced into plants without crossing, and significant progress is expected to be made in crop breeding. In particular, these methods are very effective for the breeding of outcrossing crops such as Italian ryegrass, since the genetically engineered character is transmitted to offspring dominantly. In general, these biotechnological approaches need a regenerable tissue culture system. Italian ryegrass cultivars have been bred by crossing, and each cultivar is genetically heterogeneous. Therefore, genetic variation exists not only among cultivars but also within a cultivar. This suggests that the regenerability in tissue culture varies with the genotype even in the same cultivar. Thus, the screening of the highly regenerable genotype is essential for efficient application of biotechnological techniques to this grass. Plant regeneration systems via callus culture, embryogenic cell suspension and protoplasts have been established in some Italian ryegrass genotypes by using mature seeds (Wang et al., 1993), shoot tips, leaf explants, root tips (Jackson and Dale, 1988) and immature embryos (Dale, 1980a). However, genetic variation of regeneration ability among Japanese cultivars has not been investigated in detail.

In general, mature seeds are available in all seasons, but immature embryos are only available in the flowering season. Shoot tips, leaf explants and root tips are available in all seasons if the donor plants are preserved, and suitable for an establishment of a regeneration system of Italian ryegrass. Jackson and Dale (1988) examined callus induction and plant regeneration in Italian ryegrass using root tips, shoot tips and leaf explants of in vitro-preserved 16 genotypes, but did not show data for the screening process of regenerable genotypes. Thus, we report here on: 1) screening of regenerable genotypes through tissue culture of mature seeds. 2) screening of regenerable genotypes through tissue culture of shoot
tips isolated from in vitro-preserved seedlings, and 3) variation with the genotype and explant type of callus induction and plant regeneration from the callus.

For efficient molecular breeding of Italian ryegrass, a routine and reliable plant regeneration system is indispensable. The information in the present study may be valuable for the establishment of a routine transformation system of Italian ryegrass.

### Materials and Methods

1. **Plant materials**

   Twelve commercial cultivars of Italian ryegrass (*L. multiflorum* L.) were used in the present study (Table 1). Mature seeds were treated with 50% (v/v) sulfuric acid to remove lemma, and thoroughly rinsed with distilled water. Subsequently, the seeds were sterilized with sodium hypochlorite solution (1% of available chlorine) for 20 min, and rinsed with sterile distilled water three times. The seeds were subjected to callus induction or germination in vitro.

2. **Plant preservation and propagation in vitro**

   All media used in the present study were MS medium (Murashige and Skoog, 1962) containing 3% (w/v) sucrose adjusted to pH 5.8 and solidified with 0.25% (w/v) Gelrite (Wako, Osaka, Japan). The seeds were germinated on MS medium containing 0.2 mgL⁻¹ kinetin (hereafter referred to as K₀.2 medium) under continuous fluorescent light (40 µmol m⁻² s⁻¹) at 25°C. The germinated seedlings were preserved in vitro according to the method of Dale (1980b). That is, the in vitro-germinated plants were grown and propagated aseptically on the same medium under short-day conditions (8 h light / 16 h dark; 20°C/18°C). Shoot tips (0.3-0.5 mm long) of the in vitro-germinated plants were isolated under a stereomicroscope and subjected to callus induction.

3. **Callus culture, and plant regeneration from the callus**

   Mature seeds, shoot tips (0.3-0.5 mm long), and root tips (1 cm long) were placed on callus-induction medium containing 0-7 mg L⁻¹ 2,4-D, 0-0.3 mg L⁻¹ BA and 0.5 g L⁻¹ casein hydrolysate, and cultured in the dark at 25°C. After 1 month of the culture, the induced calli were subcultured on the same fresh medium for a further month, or transferred onto K₀.2 medium to examine their regenerability. The calli induced from mature seeds were subcultured after removing the germinated shoots and roots. For regeneration, the calli were cultured under continuous fluorescent light (40 µmol m⁻² s⁻¹) at 25°C. The plants regenerated from mature seed-derived calli were maintained and propagated under the same condition for the preservation of the in vitro-germinated plants as mentioned above.

### Table 1. Callus formation from mature seeds, and plant regeneration from the callus.

| Variety  | Ploidy (n) | No. of cultured seeds (genotypes) | No. of formed calli(b) | No. of friable calli(b) | Regeneration frequency (%)(d) |
|----------|-----------|-------------------------------|------------------------|-------------------------|-------------------------------|
|          |           |                               |                        |                         | Green shoot | Albino shoot | Root           |
| Waseyutaka | 2         | 1000                          | 52 (5.2)               | 16 (1.6)                | 0.3 (3)       | 0 (0)         | 1.3 (13)       |
| Tachiwase | 2         | 960                           | 132 (13.8)             | 21 (2.2)                | 0.1 (1)       | 0.1 (1)       | 1.8 (17)       |
| Waseoaba  | 2         | 1000                          | 181 (18.1)             | 37 (3.7)                | 1.4 (14)      | 0.4 (4)       | 3.2 (32)       |
| Minamiverse | 2        | 1000                          | 84 (8.4)               | 16 (1.6)                | 0.2 (2)       | 0.6 (6)       | 1.1 (11)       |
| Yamaoba   | 2         | 1000                          | 136 (13.6)             | 18 (1.8)                | 0.2 (2)       | 0 (0)         | 1.6 (16)       |
| Minamiaoba| 2         | 982                           | 117 (11.9)             | 18 (1.8)                | 0.7 (7)       | 0.5 (5)       | 1.5 (15)       |
| Total     |           | 5942                          | 702 (11.8)             | 126 (2.1)               | 0.5 (29)      | 0.3 (16)      | 1.8 (104)      |
| Mammoth B | 4         | 1000                          | 132 (13.2)             | 10 (1.0)                | 0.1 (1)       | 0.1 (1)       | 1.0 (10)       |
| Hitachiaoaba | 4      | 950                           | 111 (11.7)             | 16 (1.7)                | 0.6 (6)       | 0.1 (1)       | 1.1 (10)       |
| Tettla    | 4         | 1000                          | 199 (19.9)             | 23 (2.3)                | 0.3 (3)       | 0.1 (1)       | 1.4 (14)       |
| Furaharu  | 4         | 425                           | 68 (16.0)              | 8 (1.9)                 | 0.9 (4)       | 0.5 (2)       | 1.4 (6)        |
| Miyukiaoba| 4         | 1000                          | 96 (9.6)               | 5 (0.5)                 | 0.3 (3)       | 0 (0)         | 0.4 (4)        |
| Meritra   | 4         | 1000                          | 130 (13.0)             | 22 (2.2)                | 1.1 (11)      | 0.1 (1)       | 1.9 (19)       |
| Total     |           | 5375                          | 736 (13.8)             | 84 (1.6)                | 0.5 (28)      | 0.1 (6)       | 1.2 (63)       |

a) Number in parentheses indicates the percentage of mature seeds that produced callus.
b) Number in parentheses indicates the percentage of mature seeds that produced friable callus.
c) Regeneration frequency (%) = [number of genotypes regenerating adventitious organ/total number of mature seeds (genotypes) cultured] × 100.
d) Number in parentheses indicates the number of friable calli regenerating each adventitious organ.
Results

1. Screening of regenerable genotypes through tissue culture of mature seeds

Mature seeds of 12 Italian ryegrass cultivars were placed onto callus induction medium containing 5 mgL⁻¹ 2,4-D. After 1 month of culture, the frequency of callus formation was investigated.

The callus formation, and regeneration from the callus were observed in all of the cultivars investigated (Table 1). In diploid cultivars, the highest frequency of callus induction was 18.1% in Waseaoba, but in tetraploid cultivars, it was 19.9% in Tetila. After a one-month subculture, these calli were divided into 2 groups: In one group, the calli were growing vigorously, yellowish and friable (hereafter referred to as friable callus, Fig. 1a). On the other hand, the slowly growing callus is overlaid with translucent, sticky and watery substance (hereafter referred to as watery callus, Fig. 1b). The two types of calli often exist in the same

Fig. 1. A friable callus induced from a mature seed (a), a watery callus induced from a root tip (b), a compact callus with fine root hair on its surface. The callus was induced from a root tip (c), and shoot formation from friable callus induced from the genotype Waseaoba No.3 (d) on the media containing 0, 0.1 or 0.3 mg L⁻¹ BA together with 5mgL⁻¹ 2,4-D. The photographs were taken after 2 weeks of culture on regeneration media.
callus, but we referred to them as friable callus, and the friable part was used for further experiments. The frequency of friable callus formation was at highest 3.7% in the diploid cultivar Waseaoba and 2.3% in the tetraploid cultivar Tetila, the mean values being 2.1% and 1.6%, respectively. The mean frequency of friable callus formation in all investigated cultivars was 1.9%.

The friable calli were cultured on K0.2 medium for one month, and the frequency of adventitious organ formation was investigated. The highest frequency of green shoot formation was 1.4% in the diploid cultivar Waseaoba and 1.1% in the tetraploid cultivar Meritra (Table 1). Albino shoots were formed at a frequency ranging from 0 to 0.6% (Table 1). Adventitious roots were formed from most of the calli cultured, and most shoots formed with adventitious roots. However, shoots were not regenerated from the calli that formed adventitious roots first. We hereafter refer to the friable callus that regenerates a green shoot as regenerable callus.

### 2. Screening of regenerable genotypes through tissue culture of shoot tips isolated from *in vitro*-germinated genotypes, and plant regeneration from the callus

In the experiment with mature seeds mentioned above, the calli of Waseaoba (diploid cultivar) and Meritra (tetraploid cultivar) regenerated green shoots at a high frequency (Table 1). Accordingly, we used these two cultivars in the following experiments.

Mature seeds of Waseaoba and Meritra were sown aseptically in K0.2 medium. Germinated seedlings (*in vitro*-germinated genotypes) were propagated aseptically, and five shoot tips from each genotype were cultured on the callus-induction medium containing 5 mg L⁻¹ 2,4-D. The numbers of tested genotypes were 63 in Waseaoba and 44 in Meritra, 107 in total.

After 1 month of culture, the induced calli were subcultured on the same fresh medium. After a further month of subculture, 88.9% and 84.1% of the genotypes from Waseaoba and Meritra, respectively, formed friable calli (Table 2). One of the friable callus was randomly selected from each genotype, and they were used in the regeneration experiment. Consequently, 22.2% and 22.7% of the genotypes from Waseaoba and Meritra, respectively, developed green shoots (Table 2). The frequency of albino shoot formation was 11.1% in Waseaoba and 4.5% in Meritra (Table 2). As in the experiment with mature seed-derived calli, adventitious roots were formed from most of the calli cultured on K0.2 medium, and shoots were not regenerated from the calli that formed adventitious roots first.

### 3. Variation with the genotype and explant type of callus induction and plant regeneration from the callus

Regenerable genotypes were detected in the experiment using mature seeds (Table 1). From these genotypes, we selected two genotypes designated as Waseaoba No. 3 and Meritra No. 8 since they formed relatively homogeneous friable callus compared with other regenerable genotypes (data not shown). These genotypes were preserved and propagated aseptically *in vitro* after regeneration from mature seed-derived calli. Shoot tips and root tips of both genotypes were planted on the media containing 0-7 mg L⁻¹ 2,4-D and 0-0.3 mg L⁻¹ BA to induce callus formation.

After 1 month of culture, the induced calli were classified into three morphological types friable, watery, and compact calli (Table 3). Shoot tips produced friable and watery calli, but root tips produced both watery calli and compact calli with fine root hair on the surface (Fig. 1a, b, c). The watery calli derived from both shoot tips and root tips grew slowly as did those derived from the mature seed. On phytohormone-free medium, some cultured shoot tips elongated (data not shown). Green or albino shoots, or both of them developed from the friable calli on the initial callus induction medium. This phenomenon was frequently observed on BA-supplemented media (Table 3).

The induced calli were transferred onto K0.2 medium to investigate their regeneration potential. After 2 weeks of culture on the K0.2 medium, green or albino shoots, or both of them developed from

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### Table 2. Callus formation from the shoot tips isolated from *in vitro*-germinated genotypes, and plant regeneration from the callus.

| Variety   | Ploidy (n) | No. of tested genotypes | No. of genotypes forming friable callus | Regeneration frequency (%)<sup>abc</sup> | Green shoot | Albino shoot | Root |
|-----------|------------|-------------------------|----------------------------------------|----------------------------------------|------------|-------------|------|
| Waseaoba  | 2          | 63                      | 56 (88.9)                              | 22.2 (14)                              | 11.1 (7)   | 87.3 (55)   |
| Meritra   | 4          | 44                      | 37 (84.1)                              | 22.7 (10)                              | 4.5 (2)    | 79.5 (35)   |

<sup>a</sup> Number in parentheses indicates the percentage of tested genotypes that produced friable callus.

<sup>b</sup> Regeneration frequency (%) = (number of genotypes regenerating adventitious organ/total number of genotypes tested) × 100.

<sup>c</sup> Number in parentheses indicates the number of tested genotypes regenerating each adventitious organ.
the friable calli (Table 3). The calli cultured on the medium with a higher BA concentration formed shoots on K0.2 medium earlier than those cultured on the medium with a lower BA concentration (Fig. 1d). No adventitious shoots emerged from the watery calli or compact calli (data not shown).

The frequency of the plant regeneration from calli was higher in Waseoaoba No.3 than in Meritra No.8. The highest frequency of friable callus formation was 100% in Waseoaoba No.3 and 80% in Meritra No.8, and the highest frequency of green shoot regeneration from the friable callus was 100% in Waseoaoba No.3 and 80% in Meritra No.8 (Table 3).

Discussion

In the present study, we observed three morphological types of calli, friable calli, watery calli, and compact calli with fine root hair on its surface (Fig. 1a, b, c). These calli were induced from mature seeds, shoot tips, or root tips (Table 1, 2, 3). Jackson and Dale (1988) have induced calli from root tips, shoot tips, and leaf explants of Italian ryegrass, and described the
callus morphology as follows: Callus cultures derived from different explants of the same genotype were morphologically similar, but there were differences in callus morphology between genotypes. They also described that the characteristics of the cultures were influenced more by genotype than by the kind of explant from which they were induced. In the present study, however, the callus morphology did not depend on genotype but on the explant type from which the callus was induced. That is, irrespective of genotype, the friable calli were induced from mature seeds and shoot tips, the compact calli from only root tips, and the watery calli from all kinds of explants tested. The morphological differences among the calli were relevant to their regeneration potential. That is, the friable calli formed green shoots on the K0.2 medium, while no adventitious shoot regenerated from other types of calli, the watery and compact calli. Thus, although the genotypic variation is crucial for tissue culture as observed by Jackson and Dale (1988), the variation with the explant type can not be neglected.

The shoot tip-derived calli obtained from in vitro-germinated genotypes regenerated adventitious organs such as shoots and roots more frequently than mature seed-derived calli. In particular, it is notable that although only 1.4% and 1.1% of mature seeds produced regenerable calli in Waseaoba and Meritra, respectively (Table 1), over 22% of shoot tips from the in vitro-germinated genotypes produced regenerable calli in both cultivars (Table 2). Thus, the shoot tips of in vitro-germinated genotypes are thought to be more effective explants than mature seeds to establish the plant regeneration system.

The type of explant and growth condition of the mother plant influence the tissue culture potential such as callus induction, protoplast yield, plant regeneration, and the ratio of green to albino shoot formation (Creemers-Molenaar et al., 1988; Takamizo et al., 1994; Olesen et al., 1995). For instance, immature inflorescences isolated from glasshouse-grown plants showed a lower percentage of albino shoot formation than field-grown ones (Creemers-Molenaar et al., 1988). These preceding reports and the present study indicate that successful screening of regenerable genotypes through tissue culture can be achieved by using appropriate kinds of explants from the plants grown under suitable conditions. The
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in vitro-preserved plant seems to be advantageous for stably providing reliable shoot tips to induce regenerable calli, because the plant is aseptically preserved under sufficient nutrition without biotic and abiotic stresses.

The present study showed that the shoot tips isolated from in vitro-preserved plants are useful for tissue culture. That is, as well as the in vitro-germinated genotypes, the regenerants from mature seed-derived calli could be preserved in vitro and could also provide suitable explant for induction of regenerable calli (Fig. 1d, Table 3). So far, these screened genotypes have been preserved and propagated in vitro by subculturing without morphological abnormality for over 5 years. This indicates that we can produce regenerable calli from the screened genotypes at any time as summarized in Figure 2.

In the transgenic experiments with Italian ryegrass, evaluation of the transgenic progenies is difficult because the progenies resulting from outcrossing are genetically diverse. The evaluation of primary transformants by clonal comparison with the same genotype from which the primary transformants are produced will lead to easy selection of an elite transformant. Accordingly, it is the best strategy for preserving the genotype for production of transgenic plant as plantlet. The present regeneration system is advantageous in this respect. Using the present regeneration system, we have already established a reliable transformation system and are routinely producing many transgenic plants (Takahashi et al., 2002).

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