Biotechnological Improvement in Timothy Breeding

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BIOTECHNOLOGICAL IMPROVEMENT IN TIMOTHY BREEDING

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Abstract

Biotechnological improvements in timothy breeding by androgenic and somatic cell culture and molecular identification were achieved. The doubled haploid (DH) plants were obtained via anther and microspore culture. A series of factors which affect androgenic embryogenesis and green plant regeneration have been evaluated. The PG-96 induction media which sharply promote androgenic embryogenesis have been established. Over 400 DH plants of timothy were obtained. Timothy somatic callus induction was successfully established using immature inflorescences and seedling stems. Suspension cultures were initiated from friable nodule-forming callus. More than 100 regenerated green plants were obtained from somatic callus and suspension clumps. DNA-based fingerprinting technologies such as RAPD, AFLP and SSR will be used in molecular identification. Appropriate primers and PCR conditions have been tested. The electrophoresis gel showed that the PCR products were informative and polymorphic. The result demonstrates that methodology based on random-primed DNA amplification can be used for timothy identification.

Keywords: *Phleum pratense*, androgenic embryogenesis, somatic embryogenesis, suspension culture, plant regeneration, molecular identification, PCR technique

Introduction

As a perennial forage grass, timothy (*Phleum pratense* L.) is one of the basic fieldcrops in global food production, and the most important grass in northern latitudes. Improved timothy cultivars have been developed. Being a hexaploid and the highly outcrossing nature of timothy, however, slows breeding of new varieties for improvements in quantitative traits like yield, protein content, digestibility. This is mainly because of the low degree of genetic homozygosity traditionally obtainable in this species, which needs selection for desirable traits to be carried out with very heterozygous material. The purpose of this study was to improve timothy plant breeding via androgenic and somatic embryogenesis and to identify the genetic background using molecular markers among timothy germplasms.

Haploid breeding is an efficient tool in comparison with conventional plant breeding in crops where self-pollination is not possible, since diploid homozygous pure lines can be obtained in a single generation by androgenic cell culture technique. This saves many generations of backcrossing to reach homozygosity by traditional means. As a perennial grass, DH timothy plants are valuable in plant breeding. Another important role of double haploids are in genome mapping where they provide excellent materials to obtain reliable information on the location of major genes and quantitative trait loci for economically important traits (Khush and Virmani, 1996).

Knowledge of germplasm diversity and of relationships among breeding materials has a significant impact on the improvement of crop plants. Several molecular marker systems including RAPD, AFLP and SSR analysis have been applied to genetic mapping and biodiversity studies.

The purpose of this study is to identify the genetic background among germplasms and to detect the variation during androgenic embryogenesis and plant regeneration using doubled haploid populations.

Material and Methods

Details of the germination and growth methods in timothy androgenic embryogenesis have been described earlier (Guo et al. 1999). The optimum developmental stage of microspore was between the very late uninucleate and the binucleate stages. A cold pretreatment of 2-4 weeks at 4 °C was used in timothy anther culture study. The PG-96 induction medium and a series of androgenic cell culture media for cereal and grass crops were tested in both the solid and liquid forms. The two different techniques employed for the mechanical isolation of microspores from timothy spikes were maceration by microblending and maceration with a pestle. After the purification, microspores were resuspended in the required amount of embryo/callus induction medium, final density of microspores was determined with a haemocytometer and adjusted to $2x10^4$ per milliliter. The cultured microspores were treated with heat shock, at 31°C for 24 h. Green plant regeneration was achieved by transferring calli to 190-2 regeneration medium (Wang and Hu, 1984) with a low level of auxin. A colchicine treatment was applied to double the chromosome complement.

For timothy somatic embryogenesis, both immature inflorescences and seedling stems were used as donor material to induce callus formation. The timothy suspension cultures were established from fast growing and small granular embryogenic calli, which were light yellow in colour.

The analysis of RAPD was performed using DNA from 10-15 plants per genotype. Total DNA was extracted from 0.1 g young plantlets. The DNA extraction method was described by Weising et al., 1995. The DNA concentration was measured by running it on agarose gels.

Results and Discussion

Timothy anther culture and microspore culture techniques were established in this study (Guo et al., 1999; Guo and Pulli, 2000). Liquid PG-96 induction medium developed, significantly promoted embryo yield; in anther culture, the best result was 800-1000 embryos (calli) per 100 anthers. In PG-96 induction medium, the NO_3^- content was adjusted to 16.5 mM and the NH_4^+ content to 2.3 mM to have a proper NO_3^-/ NH_4^+ ratio. The PG-96 medium was composed of relatively complex organic acid and vitamin compounds. For many genotypes, the PG-96 liquid medium produced a higher embryo yield, and much better results were obtained by the liquid medium than the solid medium. Compared with all other media, the PG-96 liquid medium sharply increased embryo yield in both anther culture and microspore culture.

Genotype was an important factor in androgenic embryogenesis of timothy. Embryos were obtained from 16 genotypes out of the 28 genotypes tested in anther culture and from 6 genotypes out of 12 genotypes in microspore culture. The optimum stage for microspore development was between the very late uninucleate stage and the binucleate stage. Cold pretreatment applied to the donor plants (spikes) increased embryo yield in timothy anther culture. Albinism was a serious problem in timothy androgenic cell culture but it was reduced by low light intensity conditions during regeneration in anther culture. In timothy microspore culture, macerating spikes with a blender and purificating microspores using a mannitol /maltose monohydrate interface gave a relatively high cell vital percentage. Heat shock promoted initiation of microspore culture. Over 400 green plants have been obtained thus far from anther culture and microspore culture of timothy. The doubled haploids recovered from anther culture and microspore culture were 58.5% and 65.6%, respectively.

Timothy somatic embryogenesis and green plant regeneration were successfully established using immature inflorescences and seedling stems. Somatic embryogenesis was

more successful from immature inflorescences than from seedling stems. Immature inflorescences of 0.5-1.5 cm in size cultured in MS medium containing 2 mg l^{-1} 2, 4-D gave the best performance in callus formation. Suspension cultures were initiated from friable nodule-forming callus in MS basal medium supplemented with 5.0 mg l^{-1} 2, 4-D and 0.5 mg l^{-1} ABA. The highest green plant regeneration was achieved 5-7 weeks after initiation in suspension culture.

The germplasm identification using RAPD to classify thirty-eight timothy varieties has been done in our laboratory. We have 38 genotypes of timothy from 15 countries and more than 400 doubled haploid plants of timothy from androgenic anther culture and isolated microspore culture (Guo et al., 1999; Guo and Pulli, 2000). These doubled haploid plants are very valuable for timothy breeding, but their genetic variation is unknown. In the continuation of this study, we will identify the relationship among the different cultivars and detect the variation during androgenic embryogenesis and plant regeneration using doubled haploid populations. Some preliminary results have been achieved. Appropriate primers and PCR conditions have been tested. The electrophoresis gel showed that the PCR products were informative and polymorphic. The result demonstrates a methodology based on random-primed DNA amplification that can be used for timothy identification.

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