

GENETIC SEPARATION OF ANNUAL FROM PERENNIAL RYEGRASS

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Introduction

Methods to distinguish between annual and perennial ryegrass have long been sought. Morphological characteristics that are often used to distinguish annual from perennial growth habit include rolled vs folded leaf vernalization, rapid growth, wide leaves, light color foliage, heading without vernalization, and presence of awns (Jung et al., 1996). Laboratory tests that would be more rapid than growing plants to maturity are desirable. Some of these include electrophoresis of seed proteins (Ferguson, 1984), esterase (Griffith, 1991; Griffith and Banowitz, 1992), and seedling root fluorescence (Gentner, 1929). While seedling root fluorescence has been adopted in the USA for marketing perennial ryegrass for turf, none of these laboratory tests have been readily accepted worldwide.

The Seedling Root Fluorescence Test

Seedling root fluorescence has been used as a separator to distinguish ryegrass crops for almost 60 years. Generally, germinating seedling roots of annual or Italian ryegrass fluoresce when placed under ultraviolet light, roots of perennial ryegrass do not fluoresce. Interpretation of the test, however, has been difficult since it was first implemented because of variability in the test (Nitzsche, 1960). Because of the variability, early researchers suggested that the test could only be used as an indicator of kind, but it should not be used as a rigid discriminator (Rampton, 1938). Yet, in 1990, the Federal Seed Act rules for testing seeds were amended to allow the seedling root test to be used as a variety descriptor in the ryegrasses (AOSA rules, 1994). This action made the test more rigid than originally intended because when the fluorescence level for a variety is established, any test showing values above that level are automatically classified as annual ryegrass.

One source of variation associated with the test is that some fluorescence is not readily visible (hidden) while the seedling root is still on the filter paper. It was not recognized until the rules change in 1990 that the test should have been completed by removing all seedlings from the filter paper (Colbry, 1963). For many years, roots with faint fluorescence intensity were ignored when standard or production tests were conducted. While lifting seedlings to observe faint fluorescence on the filter paper does reduce some human judgement errors in making decisions about intensity, removal of seedlings in production laboratories is time consuming.

We found that there is far more variation in the fluorescence test because of other factors than that expressed by the small contribution of not lifting roots with hidden fluorescence (Barker et al., 1997; Floyd and Barker, 1997). We provided data (Barker et al., 1999) that were instrumental for the AOSA Seed Testing Rules to be changed by eliminating lifting starting in October 1998.

It would be far better if alternative tests to seedling root fluorescence were developed based on more stable biological characteristics than fluorescence. Such tests need to be fast, inexpensive, readily available, and easy to implement.

Alternative Tests Based on DNA

The most basic way to distinguish crop kind, or even identify cultivars, would be based on genes inherent in the materials. The genetic material, DNA, is the basis of life for any organism; genes determine expression of plant traits. Genetic testing would be ideal if the exact genes that cause the difference between organisms can be identified. Knowing the differentiation genes, and where they are located on chromosomes, allows us to sequence the DNA so the building block order can be tested. Isolating DNA sequences close to the actual genes (linkage) is also beneficial to developing tests.

We have developed a DNA-based test to separate annual from perennial ryegrass (Warnke and Barker, 1998a; 1998b). The test consists of three fundamental steps that can be completed in about 24 hours. The first step is to imbibe the seeds overnight to make them soft enough to crush so DNA can easily be extracted. The second step is to introduce a master mix of chemicals containing DNA building blocks so that DNA sequences of interest can be amplified through polymerase chain reaction (PCR). The final step is simply to visualize samples that have large quantities of amplified DNA using a fluorescence dye, or other functional binding system.

Amplification, the second step, is the part of the test and the most difficult. It is difficult not because the process of amplification is hard, but because the DNA sequence most appropriate to discriminate between two genotypes must be identified. Isolation of the correct sequence takes time and considerable research effort. To be effective, the DNA sequence to be amplified needs to be part of, or closely associated to, the gene that controls the morphological separation of the genotypes. Our first example of the separation was based on first year flowering, or flowering without vernalization (Warnke and Barker, 1998a). First year flowering may not be the best genetic separation to use because there are a few perennial ryegrass plants that flower without vernalization in greenhouse tests. We believe now that photoperiod may be a more definitive separator. Annual ryegrass plants appear to be photoperiod insensitive. That

is, they will produce seed heads when day lengths are shorter than 13 hr, while perennial ryegrass plants must have more than 13 hrs. Photoperiod is somewhat complicated, but has been extensively studied in crops such as barley and wheat. There appear to be relatively few genes involved, and genetic markers linked to the more important have been identified. The genes that regulate the time of flowering are divided into vernalization response genes, photoperiod response genes, and earliness *per se* genes. Of these classes of genes only the earliness *per se* genes act in a recessive manner as occurs in annual ryegrass.

One or two major dominant genes control seedling root fluorescence, while one to four major recessive genes influence earliness. The recessive gene action of earliness in annual ryegrass makes pollen contamination difficult to identify because these recessive genes will only be expressed and visualized when in a homozygous state. Pollen contamination, therefore, will not be detected for two or more generations after contamination occurred. Genetic markers that can identify pollen contamination at the time of its occurrence will reduce annual-like genetic contamination in later generations and improve the quality of the perennial ryegrass seed crop.

While we continue our attempts to find the DNA sequences that provide the best separation, we will develop a DNA-based test that is linked to the seedling root fluorescence trait. This test, while suffering some of the same problems as the seedling root fluorescence test, will be possible to complete in about 24 hr rather than two weeks, and it will demonstrate that our concept of genetic testing to detect annual ryegrass contamination in perennial ryegrass is possible.

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