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Inheritance of superoxide dismutase (*Sod-1*) in a perennial × annual ryegrass cross and its allelic distribution among cultivars

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Abstract Identifying annual ryegrass contamination in perennial ryegrass seed lots has been of major interest in seed-testing laboratories and for seed regulatory agencies in the USA for many years. This study was conducted to characterize a superoxide dismutase locus (*Sod-1*) and determine its potential to distinguish cultivated ryegrass species. The inheritance of *Sod-1* was evaluated in a three-generation annual × perennial ryegrass mapping population and segregation fitted an expected 1:2:1 ratio for a single locus with two alleles. The molecular form of the *Sod-1* locus was determined by H_2O_2 and KCN inhibitor assays which indicated that the *Sod-1*, and a second independently segregating *Sod-2*, locus were both Cu/ZnSod enzymes. The common alleles at the *Sod-1* locus were scored in 13 annual and 24 perennial ryegrass cultivars to determine the potential of using this locus for species separation. The *Sod-1b* allele was homozygous in 98% of perennial ryegrass individuals from 24 cultivars, but those not 100% homozygous for *Sod-1b* were seed lots with unknown contamination from annual ryegrass. These results indicate that the *Sod-1b* allele in the homozygous condition is a good indicator of perenniality. All eight annual ryegrass cultivars originating in Europe or Asia had a low frequency of *Sod-1b* homozygous individuals or none at all. The five cultivars originating in the Western Hemisphere, however, had genotype frequencies for homozygous *Sod-1b* of up to 56%. The potential of the *Sod-1* locus to serve as a test to separate the two growth forms depends on the source of the annual-type contamination.

Keywords *Loliumperenne* L · *Lolium multiflorum* Lam. · Ryegrass · Superoxide disumtase · Seed testing

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Introduction

Perennial ryegrass (*Lolium perenne* L.) and Italian, or annual ryegrass (*Lolium multiflorum* Lam.) are two of the most widely cultivated grasses used for turf and forage throughout the world. These two outbred species of the *Lolium* genus are interfertile where their flowering dates overlap. In fact Naylor (1960) proposed that these two species be grouped under a common species name and be regarded as subspecies. Tyler et al. (1987) suggested that the European *Loliums* represent a hybrid swarm with perennial and Italian ryegrasses representing the extreme types. Balfourier et al. (2000) proposed that *L. perenne* evolved from a bottleneck of *Lolium rigidum* Gaud. populations. Further, *L. multiflorum* may have also arisen from the common ancestor, *L. rigidum* (Charmet et al. 1997). This common ancestry and the genomic similarities indicate that these two main species are very closely related.

Approximately 90% of the world supply of certified seed of perennial and Italian ryegrass is produced in Oregon's Willamette Valley. The close genetic similarity of the two species is of concern to seed certification agencies because genetic or physical contamination of turf-type perennial ryegrass by forage-type annual ryegrass is objectionable for high quality turf use. Identifying annual ryegrass contamination in perennial ryegrass seed lots has been of major interest in seed-testing laboratories and for seed regulatory agencies for many years. United States seed regulatory agencies utilize the seedling root fluorescence test to identify annual ryegrass contamination of perennial ryegrass seed lots. The seed-

ling root fluorescence (SRF) test is based on the finding that seedling roots of Italian ryegrass secrete an alkaloid compound called annuloline that produces a blue fluorescence under untraviolet light; however, perennial ryegrass roots do not normally fluoresce (Gentner 1929). Ever since Genter's discovery, the test has been used to determine the purity of perennial ryegrass seed samples. Nyquist (1963) was able to develop fluorescent perennial ryegrass, indicating that the fluorescent trait does not influence growth habit and is not tightly linked to any trait that conditions annuality.

The search for alternative tests for SRF has resulted in the identification of several traits associated with the annual or perennial growth type, but none have been developed into a suitable alternative to SRF. A seed esterase isoenzyme (Nakamura 1979; Payne et al. 1980; Griffith and Banowetz 1992), like the electrophoresis of other general seed proteins and isozymes, was able to detect species differences but these associations were based on bulked samples and lacked the necessary sensitivity to be an adequate replacement test (Larsen 1966; Ferguson and Grabe 1984). Morphologically, leaf vernation differences between perennial and Italian ryegrass have been used (Grabe 1998; Okora et al. 1999), but this test is time consuming and can be inconclusive. Alternatively, Charmet and Balfourier (1994) examined 13 leaf isozymes in eight *Lolium* species and *Festuca pratensis* L. Two leaf isozymes were identified that could be utilized as species indicators. A Phosphoglucose isomerase locus (*Pgi-2*) and a superoxide dismutase locus (*Sod*) showed frequency differences between *L. perenne* and *L. multiflorum*. Eight different alleles were reported at the *Pgi-2* locus with the *Pgi-d* allele much more common in *L. rigidum* and *L. multiflorum* populations than in *L. perenne* populations. The relative frequency of the *Sod-1a* and *Sod-1b* alleles of the Sod locus, originally reported to be the indophenol oxidase (*Ipo*) locus by Polans and Allard (1985), discriminated *L. perenne* from all other *Lolium* species evaluated by Charmet and Balfourier (1994).

Superoxide dismutase is a well-studied enzyme that catalyzes the disproportionation of superoxide radicals to hydrogen peroxide and dioxygen. Three forms of the enzyme exist, as classified by the metal ions present at the active site: copper/zinc (Cu/ZnSOD), manganese (MnSOD), and iron (FeSOD). These three enzymes are distributed throughout different subcellular locations, presumably because O_2^- cannot cross membranes (Takahashi and Asada 1983) and must therefore be dealt with at their sites of production. The three forms of SOD can be distinguished on electrophoretic gels by exploiting their differential sensitivities to KCN and H_2O_2 (Kanematsu and Asada 1990; Van Camp et al. 1990). Cu/ZnSOD is characterized as being sensitive to both H_2O_2 and KCN, FeSOD is sensitive only to H_2O_2, while MnSOD is resistant to both inhibitors.

The three SODs can be categorized into two families with unrelated DNA sequences. Cu/ZnSOD is located mainly in the cytosol and/or chloroplasts of plants, whereas the other family contains either Mn (MnSOD) in the mitochondria or Fe (FeSOD) in the chloroplast (Fridovich 1986). For monocotyledonous plants, cDNAs for chloroplastic and cytosolic Cu/ZnSODs and mitochondrial MnSOD have been isolated from *Oryza sativa* and *Zea mays* (Zhu and Scandalios 1993; Kernodle and Scandalios 1996; Kaminaka et al. 1997).

The objectives of this research were to: (1) study the segregation of the *Sod-1a* and *Sod-1b* alleles in an annual × perennial ryegrass mapping population, (2) establish the allelic frequencies of the *Sod-1* locus in a range of *L. perenne* and *L. multiflorum* cultivars, and (3) determine the isozyme form of SOD represented by the *Sod-1* locus.

Materials and methods

Mapping population

Segregation of SOD was conducted on a three-generation ryegrass pseudo testcross population. The population was developed by crossing one grandparent perennial ryegrass plant from cv Manhattan with an annual ryegrass grandparent plant from the cv Floregon to produce an F1 population. A second F1 population was generated by crossing a different Manhattan grandparent plant with a different Floregon plant. A random F1 clone was selected from each Manhattan maternal F1 population and these clones were crossed to produce a segregating mapping population containing 167 individuals. SOD segregation was tested on 162 progeny.

Data for time to flowering without vernalization was obtained by growing two replications of each clone of the mapping population in a growth chamber under a 24-h photoperiod (425 μmol m^{-2} s^{-1} PAR) at 25 °C for 116 days. The time of head emergence was recorded for each plant. Plants with no head emergence at 116 days were given a value of 116 days in the evaluation.

Seed source for cultivars

Seed of perennial and annual ryegrass cultivars was obtained from seed testing samples maintained by the Oregon State University Seed Laboratory, from commercial seed companies, and from Oregon Seed Certification Service voucher samples of OECD cultivars. The annual cv LE 284 was obtained from Uruguay.

Starch-gel electrophoresis

Isozyme analysis was performed on individual annual and perennial ryegrass seedlings at the three to four leaf stage of development. A crude protein extract was obtained by macerating five to six, one centimeter-long leaf pieces in 80 μl of chilled extraction buffer composed of 75 mM Tris-HCL buffer, pH 7.5, 5% polyvinyl-pyrrolidone-40 (w/v) and 14 mM mercaptoethanol (0.2% v/v). The extraction was performed in chilled, 12-sample porcelain plates with a plexiglass rod rounded on one end. The crude extracts were absorbed onto 2-mm by 8-mm blotter-paper wicks and stored at –20 °C.

Super oxide dismutase (SOD) isozymes were resolved in Tris-citrate/lithium-borate pH 8.3 gels (Wendel and Weeden 1989) after approximately 4.5 h of electric current at 50 to 60 mA. Gel slabs consisted of 10.5% (w/v) potato starch (Sigma chemical company). SOD activity stains were prepared according to Wendel and Weeden (1989). Gel slices were incubated in the dark for 30 min then placed under a light box with two 60 W fluorescent bulbs until white bands could be visualized against a dark blue gel.

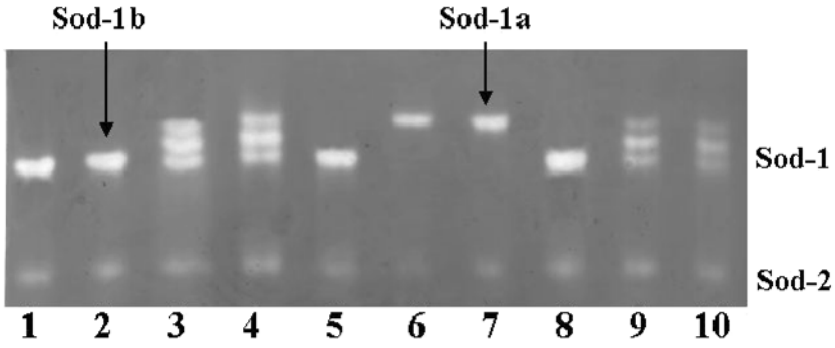

Fig. 1 12% polyacrylamide gel of superoxide dismutase from an annual × perennial ryegrass mapping population. *Lanes 1 and 2* perennial grandparents; *lanes 3 and 4* F1 parents; *lanes 5 and 8* = bb genotype; *lanes 6 and 7* = aa genotype; *lanes 9, 10* = ab genotype. The segregation ratio was 44aa:85ab:33bb, ($\chi^2 = 1.88$, $0.5 > P > 0.1$)

SOD inhibitor assays

Total protein was isolated by grinding 100 mg of leaf tissue in 300 µl of SOD activity extraction buffer (Weeden and Wendel 1989) and centrifuging for 5 min at 15,000 rpm. Protein extracts were subjected to electrophoresis at 100 V for 2.5 h in a 12% acrylamide nondenaturing PAGE. The gels were stained for SOD activity as described by Beauchamp and Fridovich (1971). Inhibitor studies were as described by Pan and Yau (1992).

Results and discussion

Three *Sod-1* genotypes were observed in the annual × perennial ryegrass mapping population (Fig. 1). The perennial grandparents from the population had the common *Sod-1b* band and the F1 parents were both three-banded heterozygotes. The annual grandparents from this population died prior to Sod genotyping; however, the fact that both F1 parents had the *Sod-1a* allele indicated that both annual grandparents must have had this allele. The segregation from 162 individuals of the mapping population was 44aa:85ab:33bb which fit the expected 1:2:1 ratio for a single locus with two alleles ($\chi^2 = 1.88$, $0.5 > P > 0.1$).

The *Sod-1* locus was inhibited by both H_2O_2 and KCN clearly indicating that *Lolium Sod-1* is a Cu/ZnSod (Fig. 2). While *Sod-1* was the primary locus a slower migrating locus marked *Sod-2* was also present (Figs. 1 and 2). *Sod-2* is also sensitive to H_2O_2 and KCN indicating that it is a Cu/ZnSod as well.

Screening for the *Sod-1a* and *Sod-1b* allele frequencies in 13 annual and 24 perennial ryegrass cultivars confirmed the findings of Charmet and Balfourier (1994). The *Sod-1b* allele is almost monomorphic in perennial ryegrass cultivars (Table 1). The most notable exceptions were 'Linn', a very old perennial ryegrass cultivar that almost meets the definition of a landrace in the USA, and Derby Supreme, a cultivar with some seed lots that have exhibited variable SRF. Absence of the heterozygous genotype (*Sod-1ab*) in Derby Supreme and Aquarius II suggested physical contamination because plants resulting from a cross between annual and perennial ryegrass would be expected to have the *Sod-1ab* genotype. Plants having the common annual ryegrass (*Sod-1a*) homozygous bands from these two cultivars, as well as those from Linn, had an annual-like growth habit (light green color and flowering without vernalization).

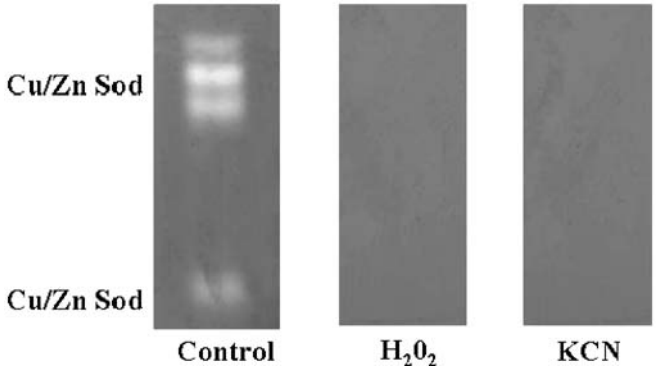

Fig. 2 Inhibitor assay of Sod-1 and Sod-2 on a 12% acrylamide nondenaturing PAGE gel. Both loci are susceptible to H_2O_2 and KCN indicating that they are Cu/Zn Sod isozymes

Most of the annual ryegrass cultivars had a very low frequency of homozygous *Sod-1bb* plants (Table 1). The reason for the low frequency of the *Sod-1bb* plants is not obvious from this research. Initial data does not indicate a selective survival advantage for any particular *Sod-1* genotype. Cultivars originating in Europe or Asia had either a low frequency of, or no, homozygous *Sod-1bb* genotypes. While we do not have the information to confirm breeding history, we suspect that European and Asian cultivars are more closely Westerwold types. On the other hand, the cultivars from the USA had homozygous *Sod-1bb* genotype frequencies ranging from 0.11 to 0.56. All of the Western Hemisphere cultivars are long-term reseeding cultivars and similar to the Italian ryegrass type (Arnold et al. 1981; Watson et al. 1990). Westerwold grasses are generally characterized as fast growing, early flowering, but not long lasting. These types originated in Norway (Jung et al. 1996). It is believed that Italian types originated in Italy (Jung et al. 1996). Our results confirm the evolutionary relationships and patterns proposed by Charmet et al. (1997) and Balfourier et al. (2000). It is interesting to note that the frequency of *Sod-1bb* plants seems to be associated with longevity. The shorter-lived Westerwolds having a low frequency of homozygous *Sod-1bb* plants, a higher frequency in the almost biennial types characterizing the Italians, and finally the perennials being almost completely homozygous *Sod-1bb* plants.

Table 1 Genotype frequencies of the *Sod-1* locus for annual and perennial ryegrass cultivars

Cultivar	Country of maintainer[a]	VFL[b]	No. of plants tested	*Sod-1* genotype		
				aa	ab	bb
Annuals						
Ace (4N)	JPN	–	19	0.32	0.68	0.0
Avance (4N)	NLD	–	20	0.30	0.70	0.0
Fabio (4N)	NLD	–	22	0.55	0.46	0.0
Jeanne (4N)	DNK	–	20	0.50	0.50	0.0
Tenor	NLD	–	20	0.30	0.70	0.0
Waseyutaka	JPN	–	24	0.50	0.46	0.04
Zorro (4N)	DNK	–	21	0.19	0.76	0.05
Aubade (4N)	NLD	–	17	0.41	0.53	0.06
Jackson	USA	98.80	19	0.31	0.58	0.11
Marshall	USA	96.00	20	0.20	0.65	0.15
Gulf (141940)	USA	99.02	43	0.16	0.61	0.23
Grazer	USA	99.78	19	0.63	0.11	0.26
La Estanzuela 284	URY	–	21	0.24	0.48	0.29
Gulf (857)	USA	99.02	40	0.23	0.45	0.33
Gulf (L19)	USA	99.02	71	0.07	0.37	0.56
Totals			396	0.28	0.51	0.21
Perennials						
Linn	USA	5.00	20	0.10	0.20	0.70
Derby Supreme	USA	2.85	16	0.13	0.0	0.88
Caddieshack	USA	1.57	11	0.0	0.09	0.91
Aquarius II	USA	–	19	0.05	0.0	0.95
Brightstar II	USA	2.24	16	0.0	0.0	1.00
Buccaneer	USA	7.44	27	0.0	0.0	1.00
Calypso II	USA	0.47	17	0.0	0.0	1.00
Cutter	USA	1.65	26	0.0	0.0	1.00
Dancer	USA	0.78	19	0.0	0.0	1.00
Delaware Dwarf	USA	2.60	15	0.0	0.0	1.00
Divine	USA	3.09	19	0.0	0.0	1.00
Express	USA	4.00	12	0.0	0.0	1.00
Lorettanova	DEU	–	11	0.0	0.0	1.00
Lowgrow	USA	1.31	23	0.0	0.0	1.00
Palmer	USA	1.04	22	0.0	0.0	1.00
Pennfine	USA	–	21	0.0	0.0	1.00
Pleasure	USA	4.09	16	0.0	0.0	1.00
Racer	USA	1.23	18	0.0	0.0	1.00
Repell III	USA	0.80	20	0.0	0.0	1.00
Saturn	USA	–	17	0.0	0.0	1.00
SRX4801	USA	–	15	0.0	0.0	1.00
Sunshine	USA	2.65	20	0.0	0.0	1.00
Top Hat	USA	0.77	16	0.0	0.0	1.00
Totals			416	0.01	0.01	0.98

[a] Country of cultivar maintainer as recorded in OECD <http://www.oecd.org/agr/code/seeds/seeds1.htm > or Oregon Seed Certification Service <http://www.oscs.orst.edu/index.html > on-line lists verified 1 August 2001. DEU = Germany, DNK = Denmark, GBR = United Kingdom, JPN = Japan, NLD = Netherlands, NOR = Norway, URY = Uruguay, USA = United States

[b] Variety fluorescence level as reported by the USA National Grass Variety Review Board

Results indicated that the *Sod-1a* allele is a good indicator of physical and genetic contamination of perennial ryegrass seed lots by Westerwold type annual ryegrass. The high frequency of *Sod-1bb* plant types in Italian ryegrass, however, would reduce accuracy if Italian ryegrass, such as the cultivar Gulf, is the source of contamination. We tested four lots of Gulf, including LE 284, which was the original progenitor of Gulf (Weihing 1963). Frequencies of the homozygous *Sod-1bb* genotype ranged from 0.23 to 0.56 for these four seed lots, demonstrating that while the frequency remained high for the cultivar, there is variability among seed lots based on the history of that particular seed source. Gulf is also the annual ryegrass cultivar longest grown in the Oregon seed production area, and the soil seed bank may contain considerable amounts of this type. The error rate of using the *Sod-1a* allele to identify physical contamination of perennial ryegrass seed lots may be as high as 50%; however, it is an excellent indicator of a problem perennial ryegrass seed lot because of the very low frequency of this allele in high quality perennial cultivars.

Homozygosity of the *Sod-1b* allele appears to be a good indicator of perenniality, and the locus is distally linked to loci influencing vernalization control of flower-

ing in the annual/perennial ryegrass mapping population (unpublished data). Hayward et al. (1994) also reported a Quantitative Trait Locus (QTL) for the date of ear emergence, the first harvest year distal to the *Sod-1* locus. These results indicate that the *Sod-1* locus, like seedling root fluorescence, is a good species indicator in the ryegrasses. The *Sod-1* locus, however, can be unambiguously scored while SRF has been shown to exhibit considerable variation due to growth conditions (Floyd and Barker 2002). The actual utility of the *Sod-1* locus as a replacement test for SRF will depend on the source of the annual contamination. The *Sod-1* locus, however, may be an excellent supplemental test to SRF for questionable seed lots.

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