

Soybean seed lustre phenotype and surface protein cosegregate and map to linkage group E

Mark Gijzen, Changren Weng, Kuflom Kuflu, Lorna Woodrow, Kangfu Yu, and Vaino Poysa

Abstract: Soybean (*Glycine max* (L.) Merr.) seeds vary in their surface properties. The lustre, or glossiness, of seeds has been classified into several different phenotypes. Soybean seeds that have a dull lustre or moderate bloom (B) may also have abundant seed surface protein, namely, an abundance of the hydrophobic protein from soybean (HPS). The seed surface protein HPS is an allergen (Gly m 1) that causes asthma in persons allergic to soybean dust. In this study, seed lustre and surface protein content are compared among 71 different soybean cultivars and lines. Dull-seeded phenotypes usually possessed abundant surface protein in comparison to shiny-seeded types, although exceptions were observed. An F₂ population of 82 individuals from a cross of OX281 (dull lustre, abundant HPS) and Mukden (shiny lustre, trace amounts of HPS) provided a basis for inheritance studies and genetic mapping analysis. Results indicate that dull seed lustre (*B*) and surface protein (*Hps*) loci are dominant Mendelian traits that cosegregate and map to soybean linkage group E. Molecular markers were used to construct a genetic map of 28 cM encompassing *B* and *Hps*. Two different molecular markers cosegregated with each of the loci. This study provides additional evidence that *Hps* may play a role in the adhesion of endocarp tissues to the seed, and offers new methods of selection for seed lustre and surface protein composition in soybean.

Key words: adhesion, allergen, hydrophobic protein, pericarp, seed coat.

Résumé : Les graines du soja (*Glycine max* (L.) Merr.) présentent une variabilité quant aux propriétés de leur surface. Cette variation phénotypique a donné lieu à une classification des graines en fonction de leur luisance. Les grains mats ou à lustre modéré (B) peuvent afficher une forte teneur en protéine de surface, la protéine hydrophobe du soja (HPS). Cette protéine HPS, présente à la surface des grains, est un allergène (Gly m 1) qui cause de l'asthme chez les personnes allergiques à la poussière de soja. Dans ce travail, la luisance et la teneur en protéine de surface ont été comparées parmi 71 cultivars et lignées du soja. Les lignées à grains mats montrent une forte teneur en protéine de surface par rapport aux lignées à grains luisants bien que des exceptions soient observées. Une population F₂ de 82 individus issus du croisement entre OX281 (grain mat, forte teneur en HPS) et Mukden (grain luisant, traces de HPS) a été employée pour des études d'hérédité et de cartographie génétique. Les résultats indiquent que le caractère mat des grains (*B*) et la présence de protéine de surface (*Hps*) sont deux caractères mendéliens dominants qui sont liés sur le groupe de liaison E. Des marqueurs moléculaires ont été employés pour définir un intervalle de 28 cM comprenant les locus *B* et *Hps*. Deux marqueurs différents montraient une co-ségrégation avec *B* et *Hps*. Cette étude apporte des évidences supplémentaires à l'effet que la protéine HPS pourrait jouer un rôle dans l'adhérence de l'endocarpe à la graine. Elle offre également de nouveaux outils pour sélectionner les phénotypes souhaités en matière de luisance des grains et de teneur en protéine de surface chez le soja.

Mots clés : adhérence, allergène, protéine hydrophobe, péricarpe, tégument.

[Traduit par la Rédaction]

Introduction

The lustre of soybean seeds is variable and seems to be primarily determined by the amount of endocarp tissue adhering to the seed surface (Wolf et al. 1981). Most soybean cultivars are classified as dull or shiny seeded, and a particu-

lar seed protein is associated with dull phenotypes (Gijzen et al. 1999). The hydrophobic protein from soybean (HPS) is an 8-KDa protein that is abundant on the surface of dull-seeded soybeans, but is present in only trace amounts on shiny-seeded cultivars. HPS is synthesized in the pod endocarp, and thus it may have a role in mediating the at-

Received 20 December 2002. Accepted 30 April 2003. Published on the NRC Research Press Web site at <http://genome.nrc.ca> on 10 July 2003.

Corresponding Editor: G.J. Scoles.

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tachment of this tissue to the seed surface. The protein is also an important allergen (Gly m 1), since it is known to cause asthma in persons allergic to soybean dust (Gonzalez et al. 1995). Severe asthma epidemics have occurred when soybean dust has blown over populated areas (Antó et al. 1989, 1993). HPS shares structural similarities to plant lipid-transfer proteins, a class of plant proteins with potential to cause food and inhalant allergies (Breiteneder and Ebner 2000).

A range of seed lustre phenotypes are observed in soybean (Juvik et al. 1989), and past studies indicate that this trait may be controlled by complementary genes. Three loci, *B1*, *B2*, and *B3*, have been proposed to account for the development of seed bloom (a heavy coating of endocarp on the seed surface) (Woodworth 1933; Guodong et al. 1987; Palmer and Kilen 1987). The *B1* locus has been placed on linkage group F (Chen and Shoemaker 1998), but *B2* and *B3* have not been mapped. However, specific genotypes that carry *B1*, *B2*, and *B3* have never been defined. Likewise, there is no description of the inheritance and mapping of dull (moderate coating of endocarp on seed surface) versus shiny (absence of endocarp) seed lustre, although it has been stated that this trait segregates in a simple Mendelian fashion with dull phenotypes being the dominant trait (Bernard and Weiss 1973). Soybean pedigree analysis has also shown that an RFLP marker from linkage group E is associated by codescent with dull-seeded phenotypes (Lorenzen et al. 1995) and that locus was referred to simply as "a *B* locus".

In this study, we further explore the relationship between seed lustre and the presence of surface protein. These traits are measured and compared in over 70 different soybean cultivars. We also examine the inheritance of seed lustre phenotype and HPS in a segregating population derived from a cross between dull- and shiny-seeded soybeans. It is shown that dull seed lustre (*B*) and surface protein (*Hps*) co-segregate and occur on linkage group E. Four different molecular markers encompassing *B* and *Hps* are placed on a genetic map spanning 28 cM.

Materials and methods

Plant materials

Soybean seeds were from the collection at Agriculture and Agri-Food Canada, Harrow, Ont. The soybean line OX281 has a dull-seeded phenotype and abundant surface HPS. This experimental line resulted from a cross of two different Harosoy isolines (Buzzell et al. 1977). Mukden (USDA germplasm PI 548391), an introduced variety originating from Manchuria, is a shiny-seeded phenotype with trace amounts of surface HPS.

Plants were grown either in field plots outdoors or in glass-enclosed greenhouses. A single F_1 plant derived from a cross between OX281 and Mukden was used to create an initial mapping population of 82 F_2 individuals. From these 82 F_2 individuals, F_3 and F_4 families were produced. Because seed coat is derived from maternal tissues, F_2 seed was used to score phenotypes for F_1 plants, F_3 seed was used to score phenotypes for F_2 plants, etc.

Determination of seed lustre and surface protein

Seed lustre was scored by visual inspection and surface protein was determined by staining with mercuric bromophenol blue or by SDS-PAGE analysis. For bromophenol blue determinations, seeds were immersed in 95% v/v ethanol containing 10% w/v $HgCl_2$ and 0.1% v/v bromophenol blue. After swirling in this solution for 30 s, seeds were rinsed twice with 95% v/v ethanol and blotted dry on filter paper. Staining intensity was recorded as heavy, moderate, light, very light, or no staining. Results from this test correlated well with seed surface HPS determinations by SDS-PAGE analysis (not shown). The test was suitable for light-colored soybeans, but did not work well for dark-colored seeds because endogenous seed coat pigments obscured the contrast necessary to assess the degree of staining. For determination of seed-surface HPS, surface proteins were extracted, analyzed by SDS-PAGE (Fling and Gregerson 1986), and visualized by silver staining (Blum et al. 1987) as described previously (Gijzen et al. 1999).

DNA extractions and molecular markers

Leaf tissues were sampled from F_2 individuals and DNA extracted according to methods described by Yu et al. (1999). Oligonucleotide sequences for simple-sequence repeat (SSR) markers were from publications (Powell et al. 1996; Cregan et al. 1999) or were downloaded from SoyBase (<http://soybase.ncgr.org/>) for synthesis by a commercial provider (Genosys Biotechnologies Inc., Woodlands, Tex.) or by the University of Guelph. One oligonucleotide from each primer pair was tailed at the 5' end with a 19-bp sequence corresponding to the M13 universal forward primer. Sequence analysis software (Gene Runner 3.05; <http://www.generunner.com/>) was used to determine the primer most suitable for tailing to reduce possible hairpin structures. The cDNA sequence encoding HPS (GenBank accession No. AF100159) was used to design a set of primers specific for this gene. The HPS forward primer annealed to a site within the open reading frame, whereas the reverse primer was from within the 3' untranslated region.

The RFLP marker A963 was provided by Dr. R. Shoemaker (USDA-ARS, Iowa State University, Ames, Iowa). A new cleaved amplified polymorphic (CAP) marker was derived from A963 to facilitate PCR-based screening methods. Primers derived from the sequence of A963 were used to amplify genomic DNA from OX281 and Mukden. The 1.4-kb amplification products then were digested with a collection of restriction enzymes to screen for polymorphisms. An *HpaII* site present in Mukden and absent in OX281 provided the basis for the codominant CAP marker, A963_CAP. Sequences and polymorphisms for all PCR-based markers that were mapped in this study are shown in Table 1 and segregation ratios are shown in Table 2.

For SSR and HPS_1 marker analysis, a sample of 25 ng of soybean genomic DNA was included in a PCR mix containing 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 10 mM Tris-HCl, 50 mM KCl, 0.0001% w/v gelatin, 0.05 μ M SSR or HPS primer pairs, 0.05 μ M M13 forward primer labeled with either IRD700 (absorption maximum 795 nm) or IRD800 (absorption maximum 685 nm) dyes (LI-COR, Lincoln, NE), and 0.4 U *Taq* DNA polymerase (Invitrogen Life Technologies, Burlington, Ont.). The PCRs were carried

Table 1. Primer sequences and polymorphisms for codominant markers associated with seed lustre and surface protein.

Marker	Sequences (5'→3')	Polymorphism mapped ^a
AT21	f: GAGAGAAAGGGACGACGATTT r: TGATGTGGTTGAAAGGTTAACTG	100/150
HPS_1	f: GGTTAGCTCCAGCAGCCACTAC r: CGATAAACAAAGATAGCGAGGAGG	576/550
A963_CAP	f: GCGGTGAATGGAAGGTGTGCT r: GTAAAAGATCCCACCCAAAGA	<i>Hpa</i> II digestion of PCR products
Sat_124	f: GGGTCCATTCCACTTTTGTACAATAT r: GGGAGTTCAAACATCCATTAGTGGTATA	255/270

Note: f, forward primer; r, reverse primer.

^aProtocols for scoring markers are outlined in Materials and methods. The sizes in base pairs (bp) of PCR products are shown for OX281/Mukden.

Table 2. Segregation of seed lustre, surface protein, and associated molecular markers in an F₂ population from a cross of OX281 and Mukden.

Locus or marker	Type ^a	Segregation ^b	χ^2 (expected)	Probability ^c
Lustre (<i>B</i>)	D	66:15	3:1	0.20
Protein (<i>Hps</i>)	D	66:15	3:1	0.20
HPS_1	CD	16:44:20	1:2:1	0.55
A963_CAP	CD	14:42:20	1:2:1	0.41
Sat_124	CD	13:40:19	1:2:1	0.39
AT21	CD	16:42:21	1:2:1	0.58

^aD, Dominant; CD, co-dominant.

^bThe total number of F₂ individuals scored for each trait or marker varies owing to missing data or sample losses.

^cProbability from a χ^2 analysis.

out in a total volume of 10 μ L, and were performed in a 96-well thermocycler (GeneAmp 9600, Perkin Elmer Inc., Foster City, Calif.) using the following program: 2 min at 94°C; 35 cycles of 45 s at 94°C, 45 s at 47°C, and 45 s at 72°C; and a final extension step of 5 min at 72°C. Reaction products were separated by electrophoresis through 6% w/v poly-acrylamide gels and products were detected by fluorescence using an automated system (LI-COR Global Edition IR² System, LI-COR, Lincoln, Nebr.).

For CAP marker analysis, PCRs contained 40 ng of soybean genomic DNA, 0.150 mM each dNTP, 2.25 mM MgCl₂, 10 mM Tris-HCl (pH 8.2), 50 mM KCl, 0.2 μ M each primer, and 1.0 U *Taq* DNA polymerase in a total volume of 25 μ L. Reactions were performed using the following program: 2 min at 94°C; 35 cycles of 12 s at 94°C, 12 s at 62°C, and 2 min at 72°C; and a final extension step of 7 min at 72°C. Duplicate samples were pooled for precipitation of DNA with sodium acetate and isopropanol. Resulting DNA pellets were washed with 70% v/v ethanol, air dried, and redissolved in 5 μ L of 10 mM Tris – 0.1 mM EDTA (pH 7.6). A sample of 1.5 μ L of purified DNA was digested with 10 U *Hpa*II (New England Biolabs, Beverly, Mass.) under conditions recommended by the manufacturer. Digestion products were resolved by electrophoresis through 1.0% w/v agarose gels and visualized by staining with ethidium bromide.

Linkage analysis

Pools of DNA for bulked segregant analysis (Michelmore et al. 1991) were prepared by combining equal amounts of

DNA from 10 dull- or shiny-seeded F₂ individuals, respectively, chosen at random from homozygous genotypes. Linkage among the markers and phenotypic traits were analyzed using computer software (MAPMAKER/EXP version 3.0b) at a LOD threshold of 3.0 and maximum distance of 30 cM (Lander et al. 1987). The Kosambi mapping function (Kosambi 1944) was used to calculate map distances.

Results

The seed lustre and surface-protein content of 71 different soybean cultivars and lines were compared to explore the relationship between these two traits. Results shown in Fig. 1 illustrate that most dull-seeded types possess heavy to moderate amounts of seed surface protein, whereas shiny-seeded types stain lightly for surface protein, or not at all. However, there were exceptions to this trend in some cultivars. We also noted that seed lustre often varied from what was reported in cultivar descriptions (not shown).

To determine the inheritance of seed coat lustre and surface protein (HPS), seed was collected from F₁ individuals and from a population of 82 F₂ plants segregating for these traits. As shown in Fig. 2, dull seed lustre (*B*) and the presence of abundant HPS (*Hps*) cosegregated as dominant, qualitative traits. All F₁ individuals produced dull seed with abundant HPS, whereas F₂ individuals could be classified in two parental categories, (i) dull with abundant HPS or (ii) shiny with trace amounts of HPS.

Fig. 1. A comparison of seed lustre and surface protein content among 71 different soybean cultivars and lines. Seed lustre was rated on a scale of 1 to 5 as follows: 1, dull; 2, intermediate; 3, somewhat shiny; 4, shiny; 5, very shiny. Values shown are means derived from scores provided by two different individuals. Surface protein was determined by staining with mercuric bromophenol blue, as described in Materials and methods.

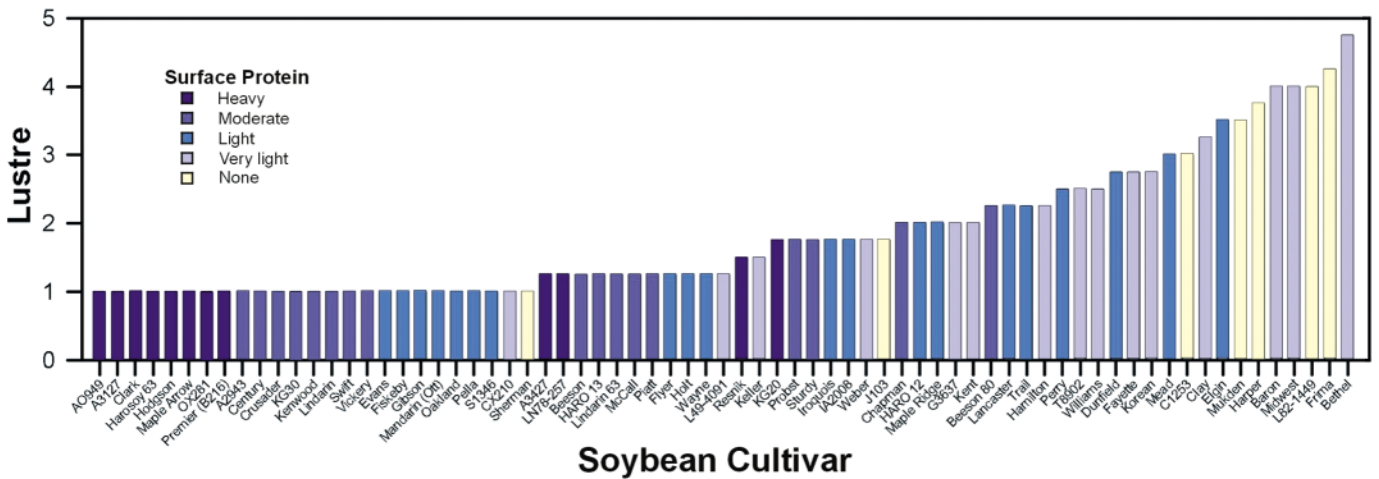
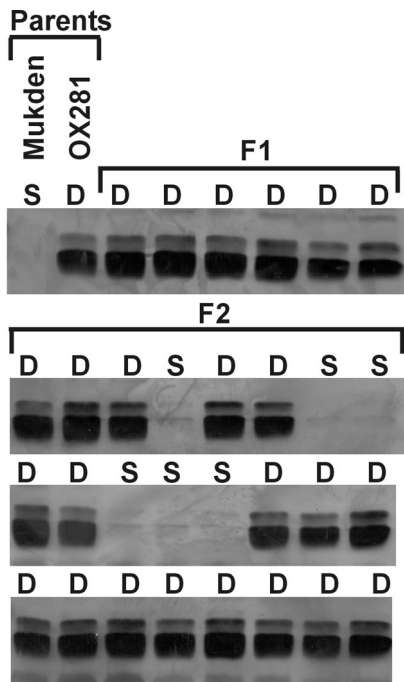


Fig. 2. Seed surface hydrophobic protein (HPS) content in parental lines, and seed from representative F_1 and F_2 individuals. Silver-stained images from SDS-PAGE analysis of seed surface extracts, showing the 8-kDa protein corresponding to HPS. Seed lustre phenotype is indicated as follows: S, shiny; D, dull.



Pools of DNA from dull- and shiny-seeded F_2 individuals were constructed for the bulked segregant analysis and were used in an initial screening of 288 SSR markers. Three primer pairs, Satt237, Satt255, and AT21, were polymorphic between the bulks. Markers Satt237 and Satt255 have previously been mapped to MLG N (Cregan et al. 1999), but no linkage information was available for AT21 (Powell et al. 1996). The entire F_2 population was then scored for each of the markers identified from the bulked segregant analysis and for an additional PCR-based marker derived from the

HPS cDNA sequence, HPS_1. The HPS_1 marker was useful because it was a codominant marker, whereas surface protein (*Hps*) segregated as a dominant trait. Results confirmed linkage for only one of the markers identified from the bulked segregant analysis, AT21. This marker was linked to *B* and *Hps*, and to the molecular marker HPS_1. The F_2 population was then screened with two additional markers from linkage group E, Sat_124 and a new marker, A963_CAP, derived from RFLP A963. These were chosen because a previous study had described an association between A963 and the seed lustre locus *B* on the basis of pedigree analysis (Lorenzen et al. 1995).

Marker A963_CAP cosegregated with *B* and *Hps* and with molecular marker HPS_1 among all of the F_2 progeny, whereas Sat_124 was only linked to these traits and markers. Shown in Fig. 3 is a map illustrating the arrangement and genetic distances among each of the markers and traits. SSR marker Sat_124 is positioned 9.6 cM from A963_CAP, HPS_1, seed lustre (*B*), and surface protein (*Hps*), whereas AT21 maps 18.4 cM to the opposite side.

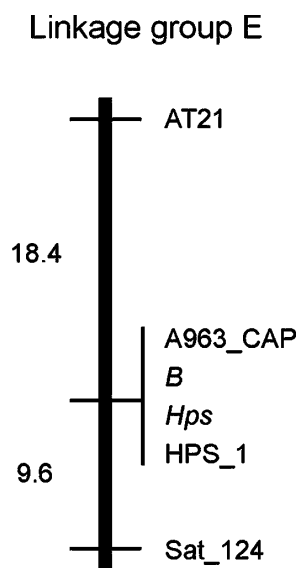
Discussion

The association of seed lustre phenotype and surface protein has been described in previous work (Gijzen et al. 1999). In this study, we have extended the analysis by comparing seed lustre to surface protein in a large number of soybean cultivars and by mapping these traits in a segregating population.

Dull-seeded phenotypes usually possess abundant surface protein. This was evident in our comparison of 71 different cultivars and lines. Exceptions to this trend were noted in this analysis and in past studies (Gijzen et al. 1999). These results demonstrate that seed bloom can develop in the absence of abundant HPS.

Although seed lustre and surface protein were scored quantitatively when comparing the 71 different cultivars and lines, we found that these traits could be assessed qualitatively in the segregating population. Seed coat lustre could

Fig. 3. A genetic map corresponding to a region of linkage group E (USDA – Iowa State University map), including traits and markers mapped in this study. The dominant traits dull seed lustre (*B*) and HPS on the seed surface (*Hps*) were mapped in relation to codominant DNA-based markers AT21, HPS_1, A963_CAP, and Sat_124 in a segregating population of 82 F₂ individuals. Values indicate distances in centimorgans (cM) as determined using the Kosambi mapping function (MAPMAKER 3.0b).



be scored readily as either dull or shiny in most cases, but there were exceptions. Further analysis of F₃ and F₄ families was useful in this regard, and increased confidence in scoring of seed lustre in a few F₂ individuals that were initially ambiguous. For example, seed from at least two F₂ plants appeared to be intermediate in lustre. The seeds were without any surface protein, suggesting recombination between these traits. However, all seed from F₃ and F₄ families derived from these individuals were shiny and without surface protein.

Attempts to quantitate seed lustre using instruments such as a gloss meter or micro-profilometer were unsuccessful or unworkable for screening large numbers of samples. Currently, the most reliable method to determine seed coat lustre is by visual inspection with a well-trained eye; however, digital scanners and image analysis software may provide a dependable alternative in the future. Caution is required in scoring this trait, since environmental effects apparently influence seed lustre. Small or immature seeds often will appear glossier than genetically identical counterparts that have fully developed and reached their normal size. Cultivars that are normally shiny seeded may also bear seeds that are quite dull, for reasons that are unclear. These factors may have influenced the results presented here, especially in our analysis of seed lustre of 71 different cultivars and lines.

Despite these caveats, we are confident in our conclusion that a major genetic locus influencing seed lustre, *B*, lies in close proximity to the molecular markers A963_CAP and HPS_1. In the cross between OX281 and Mukden, *B* and *Hps* are dominant traits that cosegregate and occur on linkage group E. Thus, our results substantiate the findings of

Lorenzen et al. (1995), since these authors found that seed lustre *B* is associated with a molecular marker from linkage group E on the basis of pedigree analysis.

Several lines of evidence indicate that *Hps* itself influences seed lustre and corresponds to the *B* locus. First, *Hps* and *B* cosegregate in F₂ populations, as we have demonstrated in this study. Second, the *Hps* genetic locus and transcript levels are polymorphic between dull- and shiny-seeded soybeans (Gijzen et al. 1999). Third, *Hps* is highly and specifically expressed in the pod endocarp, the tissue that is the primary determinant of seed lustre (Gijzen et al. 1999). Fourth, plant lipid-transfer proteins (LTPs) share structural similarities to HPS, and at least one LTP has been shown to play an important role in adhesion (Park et al. 2000). Taken together, these observations and results suggest that HPS plays a role in the adhesion of the endocarp to the seed surface and is a determinant of seed lustre. This role may be mediated by other factors for soybean cultivars with dull or bloom phenotypes and without any surface HPS. Alternate loci that have been proposed to control seed coat lustre such as *B1*, *B2*, and *B3* may provide a genetic rationale for this hypothesis.

Whether *Hps* and *B* represent a single genetic locus or two tightly linked loci may be difficult to resolve using mapping studies alone. Definitive proof may require functional analysis of the HPS protein itself. Nonetheless, the mapping of *Hps* and *B* provides new tools and information for soybean breeders and geneticists. Seed lustre is a trait that affects end uses available for seed destined for soy-based food products, since certain markets may favour one phenotype over another. The seed surface protein, HPS, is also important, since this allergen has caused severe asthma epidemics. Results described in this study demonstrate that molecular markers may be successfully applied to select for each of these traits.

Acknowledgements

The authors thank Dr. Richard Buzzell for contributions to this work; Dr. Randy Shoemaker (USDA ARS, Iowa State University, Ames, Iowa) for RFLP probe A963; Dale Anderson, Aldona Gaidauskas-Scott, Margaret Haffner, Elaine Lepp, Chuck Meharg, and Bailing Zhang for technical assistance; and anonymous reviewers for comments and suggestions.

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