Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 2408–2413, March 1996
Agricultural Sciences

GSP-1 genes are linked to the grain hardness locus (*Ha*) on wheat chromosome 5D

(*Triticum aestivum*/grain quality/inheritance/restriction fragment length polymorphism)

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Communicated by W. J. Peacock, CSIRO, Canberra, Australia, October 27, 1995

ABSTRACT **An important determinant of wheat grain quality is the hardness of the grain. The trait is controlled by a major locus, *Ha*, on the short arm of chromosome 5D. Purified starch granules from soft-grained wheats have associated with them 15-kDa polypeptides called grain softness proteins (GSPs) or "friabilins." Genes that encode one family of closely related GSP polypeptides—*GSP-1* genes—were mapped using chromosome substitution lines to the group 5 chromosomes. An F_2 population segregating for hard and soft alleles at the *Ha* locus on a near-isogenic background was used in a single-seed study of the inheritance of grain softness and of *GSP-1* alleles. Grain softness versus grain hardness was inherited in a 3:1 ratio. The presence versus absence of GSPs in single seed starch preparations was coinherited with grain softness versus hardness. This showed that grain softness is primarily determined by seed, and not by maternal, genotype. In addition, no recombination was detected in 44 F_2 plants between *GSP-1* restriction fragment length polymorphisms and *Ha* alleles. Differences between hard and soft wheat grains in membrane structure and lipid extractability have been described and, of the three characterized proteins that are part of the mixture of 15-kDa polypeptides called GSPs, at least two, and probably all three, are proteins that bind polar lipids. The data are interpreted to suggest that the *Ha* locus may encode one or more members of a large family of lipid-binding proteins.**

The wheat endosperm is a triploid tissue formed by fusion of two identical polar cells (from the female parent) with a sperm cell. The properties of the endosperm determine the end use of the wheat grain. Grain hardness or endosperm strength is one such important determinant of wheat end use. The mature wheat endosperm can be considered analogous to a polymer matrix (seed storage protein) in which a filler (starch granules) is dispersed (1). In such a structure, both the hardness of individual components and the degree of adhesion between them will determine overall strength (2). There is no detectable difference between hard and soft wheats in the individual hardness of starch granules or storage protein matrix fragments, but the degree of adhesion between starch granules and the surrounding protein matrix is higher in hard wheats (3–5). This higher adhesion results in both greater compressive strength and ductility for the endosperm as a whole (6, 7). Thus, in soft wheats, the main planes of weakness tend to occur at the starch–protein matrix interfaces and result in separation of the starch granules from the matrix with little damage during milling. As a result, starch granules from soft wheats take up less water when dough is formed and the product is more suited to the commercial production of cakes and biscuits than is dough from hard wheat flour [which is preferred for the baking of breads (8)].

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Although there are various techniques for classifying grains as hard or soft, the phenotypic difference is largely controlled by one or two genetic loci (9, 10). The major gene (*Ha*) is on the short arm of chromosome 5D (11, 12). Morrison *et al.* (13, 14) have shown that the *Ha* gene is tightly linked or identical to a gene controlling the level of extractable free polar lipids in the grain, *Fpl-1*. Genetic markers linked to milling energy in barley, which is analogous to grain hardness, were also recently shown to be located on the homologous chromosome 5H (15).

The biochemistry that determines the hardness of the grain is not known. The linkage between *Ha* and *Fpl-1* suggests that lipid metabolism or chemistry may be involved. Indeed, the integrity of amyloplast membranes in the mature endosperm of soft wheats is less than that of hard wheats (16) and this could explain the lower degree of adhesion between granules and the matrix in these wheats as well as explain the higher level of free (i.e., nonbilayer) polar lipids. In 1986, Greenwell and Schofield (17) reported the association of grain softness with a 15-kDa protein band in SDS/PAGE analyses of starch granule extracts. They suggested that the protein might act as a "nonstick" surface between starch granules and the matrix. It has since been referred to as friabilin (18) and as grain softness protein [GSP; (19)]. GSP is a mixture of at least four polypeptides, the major polypeptide(s) having a very basic pI (19–21). The basic polypeptides have been identified as puroindolines a and b and have been cloned (21, 22); a weakly basic or neutral component called GSP-1 has also been cloned (21). Minor variations in cDNA sequences have defined GSP-1-**a**, -**b**, and -**c** proteins that probably have very similar isoelectric points (21). Puroindolines a and b are known to bind polar lipids (23, 24) and because of amino acid homology between GSP-1 and puroindoline a, it is probable that GSP-1 does also (21). However, it is not clear whether these 15-kDa proteins are products of the *Ha* locus and thus direct mediators of grain softness.

In this report, we investigate the inheritance of *GSP-1* restriction fragment length polymorphisms (RFLPs), grain hardness, and the association of GSP with starch in single seeds in an F_2 population. We show that grain softness depends on seed genotype, not on maternal genotype, demonstrate that the phenotype of single seeds can be predicted on the basis of RFLPs detected with a *GSP-1* cDNA, and show that the genes for GSP-1 are on group 5 chromosomes in wheat. The results suggest that *GSP-1* genes may be useful in mapping the region of chromosome 5D in the vicinity of the grain hardness locus. The implications for the role of GSPs in grain softness are also discussed.

MATERIALS AND METHODS

Wheat Lines and Accessions. All wheat cultivars and lines used were hexaploid bread wheats, *Triticum aestivum*. Recip-

Abbreviations: GSP, grain softness protein; RFLP, restriction fragment length polymorphism.

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rocal crosses between the "hard Falcon" line and the near-isogenic "soft Falcon" line (25) were made with material grown in a temperature-regulated glasshouse at the Macquarie University (North Ryde, NSW). Subsequent generations and controls were grown in a similar glasshouse on the CSIRO site at North Ryde. Seeds of chromosome deletion stocks derived from cv. Chinese Spring (26) were provided by Robert McIntosh (University of Sydney, Plant Breeding Institute, Cobbitty, NSW) and grown at North Ryde.

Starch Preparation and Starch-Granule–Protein Extraction. Starch was prepared from single half seeds by sedimentation from suspension in 80% (wt/vol) aqueous CsCl as described by South and Morrison (27), except that seeds were initially crushed by hammering them between steel plates. Proteins associated with the purified starch granules were extracted with SDS/PAGE sample buffer and separated on reducing SDS/polyacrylamide gels as described by Jolly et al. (19) and then silver stained using a kit (BioRad) according to the manufacturer's instructions. The presence or absence of 15-kDa protein (GSP) was then scored. A faint 15-kDa band was scored as negative for GSP, since water-washed starches from hard wheats often have trace amounts of GSP or 15-kDa α-amylase inhibitor subunits on them (17, 19).

Preparation and Analysis of Plant DNA. DNA was extracted from the leaves or immature inflorescences of wheat plants as described by Appels and Moran (28). After digestion of DNA with restriction enzymes for 4–18 h, it was electrophoresed in agarose gels in 1× TAE buffer (29) and then blotted by capillary action using 0.4 M NaOH onto Hybond-N^+ membranes (Amersham) for 6 h. After blotting, membranes were rinsed twice in 3× standard saline citrate [SSC (29)]/0.1% SDS and blotted dry with a sheet of 3MM paper (Whatman). Prior to hybridization with α-^{32}P-labeled probes, blots were preequilibrated with hybridization mixture (30) at 42°C for 6–20 h. Probes were synthesized by random hexanucleotide priming from gel-purified plasmid inserts (31) and separated from unincorporated [α-^{32}P]dATP (Bresatec) using Sephadex G50 (Pharmacia) spin columns (29). The prehybridization mixture was removed and replaced with fresh mixture containing heat-denatured DNA probe at $\approx 10^6$ cpm/ml and hybridization proceeded at 42°C overnight. The blot was washed two times for 30 min in 2× SSC/0.1% SDS at room temperature and then two times for 10 min in 0.5× SSC at 65°C. Autoradiography was performed at −60°C with XAR-5 film (Kodak) for as long as required.

Analysis of Hardness, GSP, and RFLP Phenotypes in an F_2 Population. Near-isogenic hard Falcon [Symes' (25) Heron/7*Falcon Hard] and soft Falcon [Symes' (25) Heron/7*Falcon Soft] lines were crossed to produce 31 F_1 seeds heterozygous at the single locus (9, 25) that segregates for grain softness in these lines. Flowers on the germinated F_1 plants were left to self-fertilize to produce an F_2 generation of seed segregating for softness alleles on a near-isogenic (Falcon) background. The mature F_2 seed was divided transversely to produce an "embryo half" and a "brush half." The seed hardness of individual brush halves was measured as the S_{max} by the endosperm cylinder compression method (7). The original F_1 seeds were not tested, since their grain filling during ripening had been inadequate. After the hardness testing, the GSP associated with starch purified from the brush half was determined by SDS/PAGE. The embryo seed halves were planted to produce F_2 plants and DNA extracted from these plants was subjected to DNA blot analysis to detect RFLPs described below. Individual F_3 seeds from selected F_2 plants underwent further hardness testing to confirm the genotype of the F_2 material. This experimental strategy is described in Fig. 1.

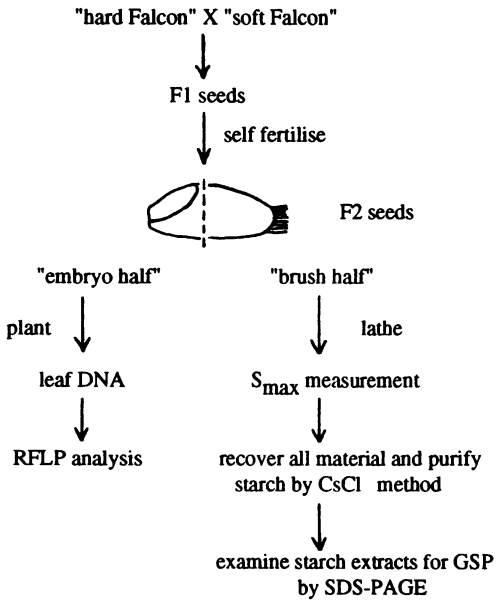

FIG. 1. Design of an experiment using near-isogenic lines derived from cultivars Falcon and Heron (25) to test at the single seed level for cosegregation of softness with the presence of GSP on washed starch and with *GSP-1* RFLPs.

RESULTS

Analysis of the Segregation of *Ha* Alleles at the Single Seed Level. Previous studies of the inheritance of wheat endosperm hardness (9, 10, 12, 32–35) have used seed pooled from individual plants to measure softness by milling, because no reliable single seed test was available. In such studies, it has been impossible to determine whether hardness variation is genetically controlled directly at the seed level or indirectly at the maternal level, because it was difficult to obtain enough F_1 seeds for milling. The distinction is not trivial, but it has not been previously addressed in the literature. Thus, an experiment was designed to resolve this issue (Fig. 1). Segregation at the *Ha* locus and the relationship between grain softness and the occurrence of GSP in extracts of starch granules was analyzed using an F_2 population of 43 plants. The hardness phenotype of individual seeds was determined by compression analysis (7) and the presence of GSP in extracts of purified starch from the same seeds was determined by SDS/PAGE (e.g., Fig. 2*A*). The relationship between hardness and the presence of GSP in starch granule extracts is shown in Table 1 and Fig. 2*B*. Generally, grain hardness and absence of GSP cosegregated. However, for a few F_2 plants, such as b11 and f1, there seemed to be identical hardness scores but clear differences in the amounts of GSP extractable from the starch. These plants, and others, were taken to the F_3 generation and it was demonstrated that they were, for example, homozygous hard and soft, respectively. Their initial identical hardness scores had probably been due to flaws in the endosperm cylinder sample. The greater spread of hard and soft scores in the F_3 plants was probably due to segregation of minor genes (9) that influence hardness. The soft/hard segregation fits a Mendelian 3:1 ratio ($X^2 = 0.38$; $0.7 > P > 0.5$; $n = 43$), indicating that single-locus inheritance is the best model. This unequivocally showed that the hardness of a seed is determined by expression of genes in the seed during its development and not by the supply of factors to the seed by maternal tissue, as in the latter case no segregation would have been visible in the F_2 seeds. The dominance of the soft *Ha* allele from cv. Heron over the hard *ha* allele from cv. Falcon was almost complete since no clear heterozygous phenotype was apparent. In previous studies using these lines, the heterozygous phenotype has been separable from the homozygous phenotype (9), but this has presumably been because the pooled soft and hard seeds from heterozygous plants gave an intermediate hardness score when milled. No starches purified from hard seeds

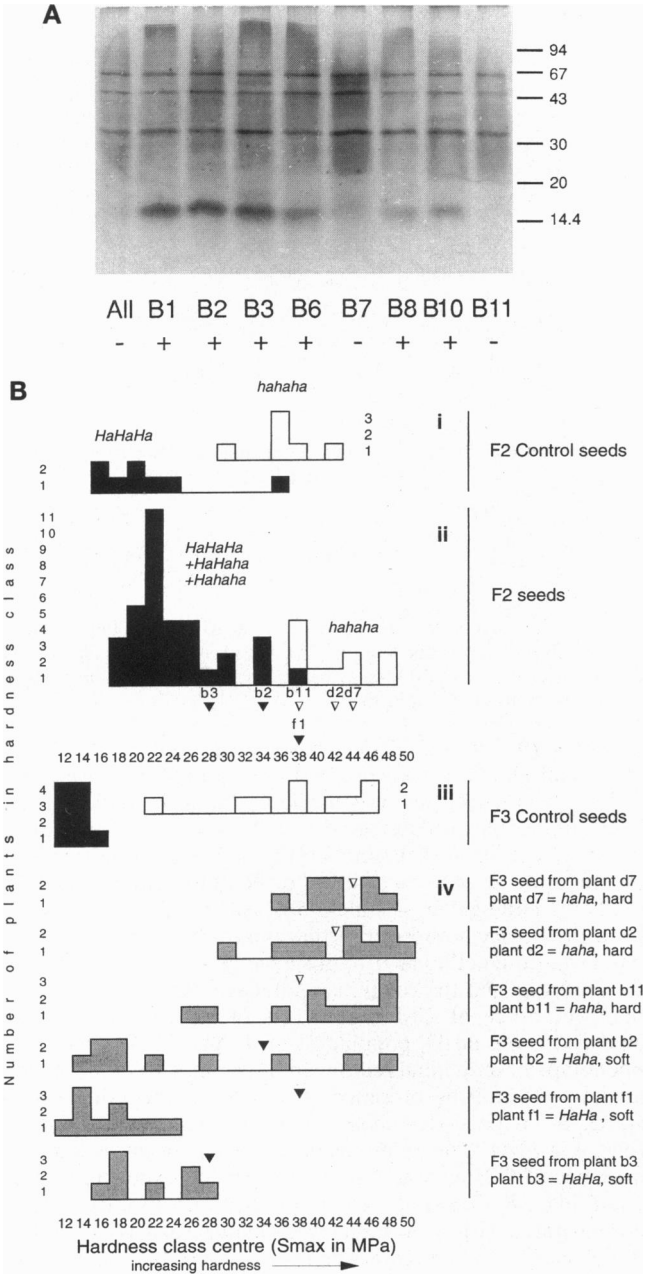

FIG. 2. Cosegregation of grain softness with the presence of GSP in purified starch extracts in an F_2 population. (*A*) Example of SDS/PAGE of starch granule proteins from F_2 seeds. Each extract is from an F_2 segregate (a11, b1, b2, etc.) of crosses between hard Falcon and soft Falcon. Extracts of starch purified from half seeds were fractionated by reduced SDS/PAGE and silver-stained. The relative mobility of protein standards is shown on the right in kDa. Lanes +, GSP readily detected; lanes −, little or no GSP detected. (*B*) Segregation of seed softness and the presence of GSP in starch extracts in the F_2 population. Note that the endosperm is a triploid tissue with two doses of identical alleles from the female parent and one dose from the male parent. The embryo and plants grown from it are diploid. Black, GSP readily detected in starch extracts; white, little or no GSP detected in starch extracts; hatched, GSP content of starch extracts not determined. (*i* and *iii*) Parental line controls. ■, Soft Falcon; ®, hard Falcon. (*ii*) F_2 segregates. (*iv*) F_3 segregates. Known or inferred genotypes of the endosperms are given in *i* and *ii*, while the known genotypes of the embryos (i.e., resulting plants) are given in *iii* and *iv*. ▼ or ▽, F_3 population was grown from an individual F_2 plant (identified above the pointer) to confirm the genotype of the F_2 plant. (*iv*) Genotype and phenotype of the selected F_2 plant, deduced from F_3 segregation, is given.

Table 1. Segregation of grain hardness and starch-associated GSP in F_2 seeds

Type of grain	High GSP	Low GSP
Soft	34	0
Hard	0	9

had readily detectable GSP, while all starches purified from soft seeds did have readily detectable GSP associated with them. Alleles controlling softness and the occurrence of GSP in purified starch granule preparations are therefore linked or identical because their segregation was completely dependent in the population examined. This was as expected from previous studies of GSP (17, 19, 36). The data demonstrated that the presence of GSP in extracts of starch purified from single half seeds is a simple and reliable method for determination of hardness genotype. It was, in fact, more reliable than directly measuring single seed hardness, presumably because hardness can be influenced by environmental factors (6).

Mapping of GSP-1 Genes to the Short Arm of Chromosome 5D. We have previously described a cDNA family (*GSP-1a*, *-b*, and *-c*) that encodes components of the complex of 15-kDa proteins called GSP (21). We carried out a series of DNA blot analyses with a *GSP-1a* cDNA probe [SR3.1 (21)] to investigate the relationship between *GSP-1* genes and the *Ha* locus. The *GSP-1* genes were unambiguously mapped to the group 5 chromosomes using DNA from the chromosome deletion lines (derived from the soft cultivar Chinese Spring) developed by Sears (26) (Fig. 3). The genes on chromosome 5D were all on the short arm.

Detection of GSP-1 RFLPs. Digestion of DNA with *Pvu* II and/or *Bam*HI produced DNA blot patterns much more complex than digestion with *Eco*RV (Figs. 4 and 5). Under the stringency conditions used, the *GSP-1* probe hybridizes only to *GSP-1* cDNA clones and not to puroindoline cDNAs (C.J.J., unpublished data), so the differences in intensity between various bands was very likely due to differences in *GSP-1* sequence copy number and not to differences in sequence identity to the probe. In addition, *GSP-1* genes, like most seed storage protein genes, are intronless (S.R., unpublished data). Since all the known *GSP-1* cDNA clones have no internal *Bam*HI sites and only one has a *Pvu* II site close to the 5′-extremity of the *GSP-1* probe (21), each *GSP-1* gene very

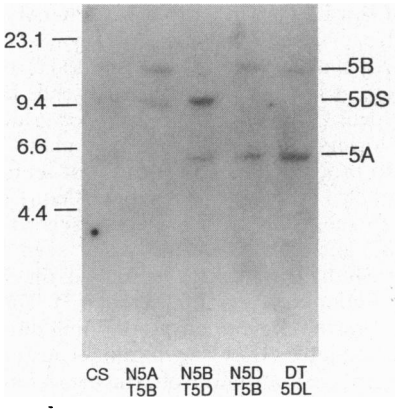

FIG. 3. DNA blot analysis (using 3.5 μg of total nucleic acid per lane) of wheat DNA digested with *Eco*RV. Wash stringency was 0.2× SSC/0.1% SDS at 65°C and autoradiography was for 24 h. Lanes: CS, cultivar Chinese Spring; NT5A5B, CS, nullisomic for chromosome 5A, tetrasomic for chromosome 5B; NT5B5D, CS, nullisomic for chromosome 5B, tetrasomic for chromosome 5D; NT5D5B, CS, nullisomic for chromosome 5D, tetrasomic for chromosome 5B; DT5DL, CS, ditelosomic for the long arm of chromosome 5D (i.e., no short arm of chromosome 5D present). Relative mobility of DNA standards (in kbp) is shown on the left and the chromosomal origin of bands is shown on the right.

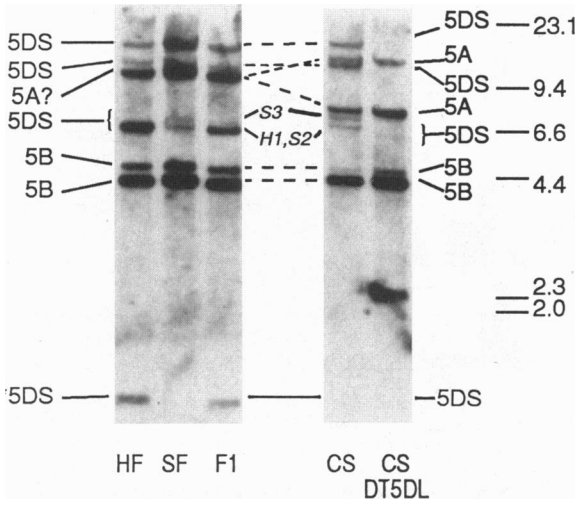

FIG. 4. DNA blot analysis of wheat DNA digested with *Pvu* II. DNA blot analysis was as described in Fig. 3, except wash stringency was 0.5× SSC/1% SDS at 70°C and autoradiography was for 2 weeks. Lanes are as in Fig. 3 plus HF, hard Falcon; SF, soft Falcon; F_1, (HF × SF)F_1 hybrid (see Fig. 1). Relative mobility of DNA standards (in kbp) is shown on the right and the chromosomal origin of bands is shown on the left and right. RFLP bands *H1* (detectable in HF and F_1, *S2* (detectable in SF and CS), and *S3* (detectable in SF, F_1, and CS) are identified. Known or probable chromosomal origin of the bands marked as 5A, 5B, and 5DS was based on Chinese Spring chromosome deletion lines. A 1.45-kbp band that produced an intensity RFLP between HF and SF is marked with an asterisk.

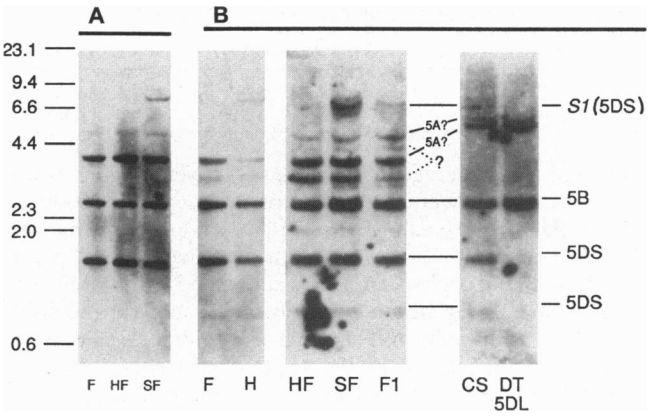

FIG. 5. DNA blot analysis of wheat DNA digested with *Bam*HI (*A*) or with *Bam*HI and *Pvu* II (*B*) DNA blot analysis (using 15 μg of total nucleic acid per lane) was as described in Fig. 3, except that autoradiography was for 90 h. Lanes are the same as for Fig. 4, plus F, Falcon; H, Heron; HH, hard Heron; SH, soft Heron. RFLP *S1* (detectable in H, SF, and CS) and the known or probable chromosomal origin of bands are indicated on the right. Relative mobility of DNA standards (in kbp) is shown on the left.

probably produced only one band in the DNA blots. Of course, each band could represent more than one gene.

Digestion of DNA with the endonucleases *Bam*HI and/or *Pvu* II produced some variant fragments (named *S1*, *S2*, *S3*, and *H1*) encoding GSP-1, when the hard cultivar Falcon was compared to the soft cultivar Heron or when near isogenic soft and hard cultivars derived from them were compared (Figs. 4 and 5). In *Pvu* II digests (Fig. 4), the 7.2-kbp fragment seen in hard Falcon DNA (*H1*) was replaced by two less-intense fragments of 7.2 kbp (*S2*) and 7.9 kbp (*S3*) in the near isogenic soft Falcon DNA. Hard Heron and cv. Falcon (hard) produced RFLP patterns similar to hard Falcon, while soft Heron and cv. Heron (soft) produced RFLP patterns similar to soft Falcon (data not shown). *S2* and *S3* could be detected only when the autoradiograph was exposed for much longer times than was necessary to detect *H1*.

In *Bam*HI digests, a difference in intensity of the 9.4-kbp fragment was detected in the near isogenic lines soft Falcon and hard Falcon (Fig. 5*A*) and soft Heron and hard Heron (data not shown). Again cv. Heron and cv. Falcon were similar to soft Falcon and hard Falcon, respectively (data not shown). The 9.4-kbp fragments common to DNA from these lines were cut into smaller fragments by subsequent digestion with *Pvu* II, leaving a 9.4-kbp fragment (*S1*) that was unique to cultivar Heron and the soft lines derived from it (Fig. 5*B*; data not shown). Although *S1* was seen as a unique band only when *Bam*HI and *Pvu* II restriction were combined (Fig. 5*B*), it was probably produced by restriction with *Bam*HI alone. Band *H1* was at least three times as intense as bands *S1*, *S2*, and *S3* (Figs. 4 and 5), suggesting that it contained 3 times as many *GSP-1* sequences. Comparison with DNA blot patterns from the soft cultivar Chinese Spring confirmed that fragments *S1*, *S2*, and *S3* were encoded by the short arm of chromosome 5D (Figs. 4 and 5). This placed them on the same chromosome arm as the *Ha* locus (11, 12). A band equivalent to RFLP *H1* was not detectable in Chinese Spring and therefore could not be mapped using the Chinese Spring lines (Fig. 4). Bands *S1*, *S2*, *S3*, and *H1* should all have been apparent in appropriate digests of DNA from F_1 plants, but, in practice, band *S2* was masked by *H1* (since they were the same size) and band *S3* was very difficult to detect in heterozygous plants because of the proximity of band *H1*/*S2* (e.g., Fig. 4).

Cosegregation of *GSP-1* Alleles with *Ha* Alleles in an F_2 Population. The results above suggest that recombination between softness or hardness alleles and the RFLP fragments *S1*, *S2*, *S3*, and *H1* was low during the breeding of the near-isogenic lines from the parental cultivars Falcon and Heron. To investigate the degree of linkage between *GSP-1* RFLPs and the *Ha* locus, a RFLP analysis of the F_2 population from a cross between soft Falcon and hard Falcon was carried out. The embryo-containing halves of F_2 seeds shown in Fig. 2 were planted to produce F_2 plants. DNA extracted from these plants was subjected to DNA blot analysis using insert SR3.1 as a probe. Bands *S2* and *S3* were not followed in the population for the reasons given above. The 44 F_2 plants analyzed could be separated into three types based on their *S1* and *H1* RFLP patterns (Table 2 and Figs. 6 and 7). Type 1 plants produced only the hard parental RFLP band, *H1* (*H1*/−); type 2 plants produced only the soft parental RFLP band, *S1* (−/*S1*); and type 3 plants produced both RFLP bands, *S1* and *H1* (*H1*/*S1*), and the intensity of both bands was reduced. As expected, the F_1 heterozygotes fitted the third group (Figs. 4 and 5). None of the 44 plants examined were null for both *H1* and *S1*; therefore, these two RFLPs acted as if allelic, or at least linked. Like band *S1*, band *H1* is therefore also encoded by the short arm of chromosome 5D. In the cases where bands *S2* and *S3* were detectable, they were coupled with band *S1* (data not shown).

The brush halves of the seeds producing the −/*S1* and *H1*/*S1* plants had already been classified as soft by S_{max} measurement and by the ready detection of GSP in starch extracts, while the brush halves of the seeds producing the *H1*/− plants were all classified as hard by these two tests (Figs. 2 and 7). The implied classification of the *H1*/−, *H1*/*S1*, and −/*S1* plants into *haha*, *Haha*, and *HaHa* genotypes, respectively (Table 2 and Fig. 7), fits a Mendelian ratio of 1:2:1 (χ^2 = 1.95; 0.5 > P > 0.25; n = 44). Because the maternally donated alleles present in the zygote are duplicated in the endosperm (reviewed in ref. 37), the triploid endosperms in the seeds that produced the *H1*/− plants were *hahaha*, the *H1*/*S1* endosperms were *HaHaha* or *hahaHa*, and the −/*S1* endosperms were *HaHaHa*. The zygotes would of course have had the same diploid genotypes as the mature plants that they produced. Band *S1* cosegregated with the dominant *Ha* allele,

Table 2. Segregation of grain hardness and RFLPs in F_2 seeds

Type of grain	RFLP $-/S1$	RFLP $H1/S1$	RFLP $H1/-$
Soft	16	19	0
Hard	0	0	9

and band *H1* cosegregated with the recessive *ha* allele. It can be seen that the average softness of the seed that produced *H1/S1* plants (*HaHaha* or *Hahaha* in the endosperm) was only slightly less than that of the seed that produced $-/S1$ plants (*HaHaHa* in the endosperm). This confirmed that the softness allele from cultivar Heron is largely dominant over the hardness allele from cultivar Falcon. This dominance allows the phenotype segregation in the triploid endosperm to fit a classical diploid 3:1 ratio because the doubling of the maternal allele has little or no effect upon phenotype—that is, *Hahaha* was phenotypically identical to *HaHaha.*

One *GSP-1* RFLP Segregated Independently of the *Ha* Locus. A RFLP based on a band intensity of 1.45 kbp was observed between hard Falcon and soft Falcon (Fig. 4, *). This band was mapped to the short arm of chromosome 5D in Chinese Spring (Fig. 4). However, this difference was not observed in the pair of near-isogenic Heron lines or in the Falcon and Heron parents (Fig. 4; data not shown). Furthermore, in the segregating F_2 population, this band was of equal intensity in all plants (data not shown). Therefore, although the band did produce an intensity RFLP, this RFLP was unique to the hard Falcon and soft Falcon pair. The hard Falcon versus soft Falcon RFLP was probably due to a crossover that occurred in one of the last backcrosses before soft Falcon and hard Falcon were established as pure lines (25). The crossover did not persist in linkage with the *Ha* locus in the Heron lines or in the hard Falcon cross soft Falcon F_2 plants, indicating that the *GSP-1* alleles involved were not closely linked to the *Ha* locus. This RFLP demonstrated that at least one *GSP-1* gene on chromosome 5D could segregate independently of the *Ha* locus.

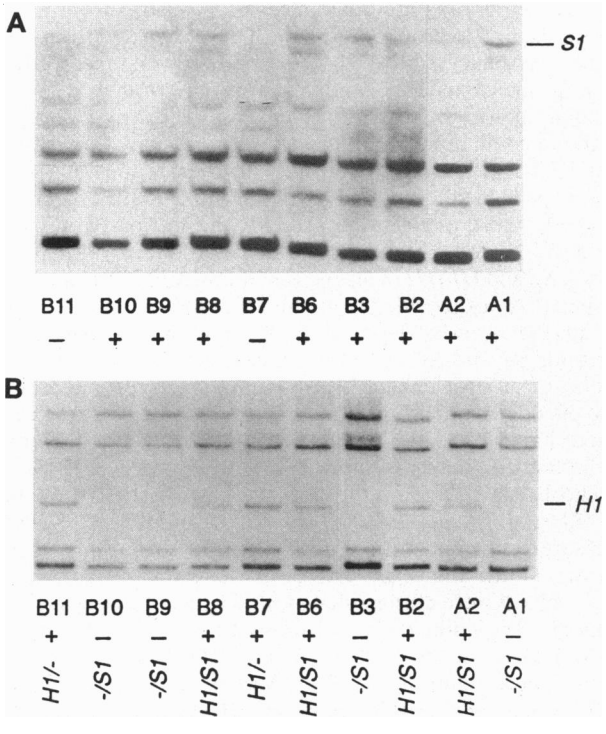

FIG. 6. Representative DNA blot analyses of wheat DNA from 10 F_2 plants (a1, a2, b1, b2, etc.) derived from crosses between hard Falcon and soft Falcon (see Fig. 1). DNA was digested with *Pvu* II + *Bam*HI (*A*) or *Pvu* II only (*B*). DNA blot analyses (using 15 μg of total nucleic acid per lane) were as described in Fig. 4, except that autoradiography was for 4 days. Lanes: +, RFLP *S1* (*A*) or *H1* (*B*) detected; –, RFLP *S1* (*A*) or *H1* (*B*) absent.

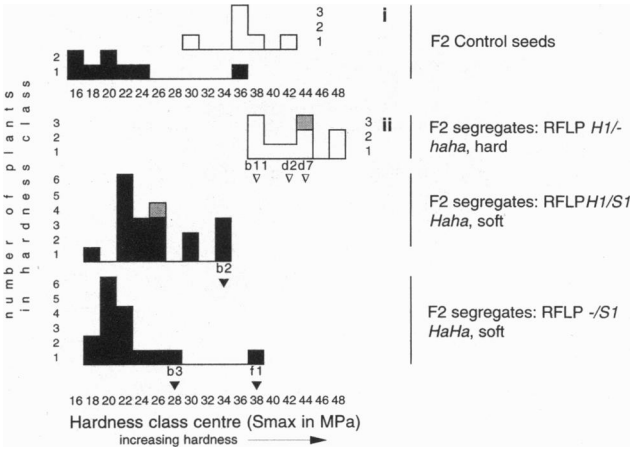

FIG. 7. Segregation of seed softness and hardness with RFLP types (e.g., Fig. 6) in an F_2 population. This material descended from crosses between hard Falcon and soft Falcon as described in the text and in Figs. 1 and 2. Note that the endosperm is a triploid tissue with two doses of identical alleles from the female parent and one dose from the male parent. Embryo and plants grown from it are diploid. Black, GSP readily detected in starch extracts; white, little or no GSP detected in starch extracts; hatched, GSP content of starch extracts not analyzed. (*i*) Parental line controls. ■, Soft Falcon; ®, hard Falcon. (*ii*) F_2 segregates. ▼ or ▽, F_3 population was grown on from an individual F_2 plant to confirm genotype as shown in Fig. 2*B*.

DISCUSSION

We have previously reported on the isolation of three closely related *GSP-1* cDNAs from a soft Falcon library (21). cDNAs encoding the other major components of the GSP complex—namely puroindoline a and b—were characterized by Gautier *et al.* (22). One of us independently isolated puroindoline a cDNAs from soft Falcon and Timgalen libraries using insert SR3.1 as a probe and using low stringency hybridization conditions. Puroindoline b cDNAs were similarly isolated using one of the puroindoline a cDNAs as a probe (C.J.J., unpublished data). This work showed that under the high-stringency conditions used in this report, only *GSP-1* sequences would be detectable with insert SR3.1. Consistent with the isolation of three different *GSP-1* cDNAs, we report here the detection of a number of *GSP-1* genes in wheat. All of the *GSP-1* genes were unambiguously mapped to group 5 chromosomes. The genes on chromosome 5D are on the short arm and it is therefore likely that those on 5A and 5B are also. Furthermore, the *GSP-1* genes on chromosome 5D are clustered since they segregated as a single locus at the extreme distal end of the short arm in F_2 populations of *Triticum tauschii* (38). In this study, we show that at least two *GSP-1* alleles (*H1* and *S1*) are linked to the wheat hardness locus, *Ha*. A *GSP-1* allele at a locus defined by RFLP *S1* and probably also alleles at loci defined by RFLPs *S2* and *S3* cosegregated with the softness allele *Ha*, while RFLP *H1*, which possibly identifies alleles at the same three *GSP-1* loci, cosegregated with the hardness allele, *ha*. A 1.45-kbp *Pvu* II RFLP did not cosegregate with grain hardness differences. Thus, some *GSP-1* genes on the short arm of chromosome 5D are more tightly linked to the *Ha* locus than others. No recombination between RFLP *S1* and *Ha* was detected in the 44 F_2 progeny examined (Table 2 and Fig. 7). It is clear that the marker *S1* is linked to softness (significance probability, < 0.001). However, because of the small numbers examined there could still be a maximum genetic distance of 8.4 centimorgans (cM) between RFLP *S1* and *Ha* (at the one-sided 95% confidence limit) and further experiments are required to refine the

estimate. Backcrossing of an F_1 to a homozygous recessive (hard) plant is usually a better way to identify recombinants than analysis of an F_2 population. Backcrossing was not used in this study, because it was found that seeds produced from manual fertilizations were not adequate for single seed hardness testing. However, now that it is established that GSP in purified starch extracts can be used as an unambiguous marker for softness in single seed studies, a backcross population could be analyzed using GSP content as the marker for softness.

RFLPs *S1*, *S2*, and *S3* probably encode a single *GSP-1* gene each and may each be allelic with one of three fragments that make up RFLP band *H1*. If this is correct, then chromosome 5D contains ≈6 *GSP-1* loci, and the complete *T. aestivum* genome contains ≈18. The soft-specific RFLPs (*S1*, *S2*, *S3*) encoded in lines derived from cultivars Falcon and Heron were also detected in the unrelated soft cultivar Chinese Spring (Figs. 4 and 5). This implies that the *GSP-1* RFLPs may be widespread in wheat cultivars, but this should be investigated further. The consistent correlation of hardness genotype and RFLPs detected with *GSP-1* gene probes in a range of unrelated cultivars would be further evidence that *GSP-1* alleles are part of the *Ha* locus. Immunoblotting with an antiserum raised against a puroindoline N-terminal peptide showed that puroindoline accumulation is, like GSP-1 accumulation, dependent on the short arm of chromosome 5D (21). Thus, the puroindoline loci may also be linked to the *Ha* locus.

GSPs or friabilins were originally considered to be linked to hardness on the basis of their association with starch granules from soft wheats (17). Since then there have been contradictory reports of *in situ* localization based on immunofluorescence of friabilin on endosperm starch granules of both soft and hard wheats (39) and of puroindolines in the globoid of aleurone cells (22). Gautier et al. (22) have suggested that the presence of puroindolines (which are the major components of GSP or friabilin) on water-washed starch granules is therefore casual as a mechanical consequence of the formation of complexes between puroindolines and starch-associated lipids during dough mixing. After dough formation, high levels of GSP are associated with starch granules from both soft and hard wheats but water-washing removes it more effectively from hard wheats (19). If the association of GSP with soft wheat starch granules is consequential to the primary effect of the *Ha* locus, then it is a remarkable coincidence that the genes for GSP proteins are linked to the *Ha* locus. Differences in amyloplast membrane integrity (16) and in lipid extractability (13, 14) have been noted between hard and soft wheat endosperms. Puroindolines are known to bind membrane lipids (23, 24) and homology between GSP-1 proteins and puroindolines suggests that GSP-1 proteins may also specifically bind membrane lipids (21). Differences in expression, sequence, or distribution of GSP-1 and/or puroindolines could conceivably affect lipid distribution or membrane integrity in the desiccating seed enough to produce changes in the final endosperm texture. Rather than being a single gene, the *Ha* locus may be complex and consist of many lipid binding proteins so that differences in any or many of these genes result in different *Ha* alleles. This would be consistent with the finding by Chalmers, et al. (15) that the major milling energy (equivalent to hardness) locus in barley was a quantitative trait locus spanning ≈13 cM.

We suggest the following hypothesis. The *Ha* locus consists of many genes encoding lipid binding proteins of different functions. Depending on which of these proteins are expressed and their amounts, the membrane structure is altered in the endosperms, producing soft or hard wheats. This change in membrane structure is also reflected in the artefactual binding of differing amounts of GSP to the starch granule. This model is consistent with (*i*) the initial observation that starch granules isolated from soft wheats contain GSP, (*ii*) that both soft and hard wheats contain GSP, and (*iii*) the linkage of GSP-1 genes and the *Ha* locus. Further experiments are needed to test this hypothesis.

We are grateful to Dr. Robert McIntosh (University of Sydney, Plant Breeding Institute, Cobbitty, NSW) for teaching C.J.J. how to cross wheat plants and for providing chromosome deletion lines. This work was funded by grants to S.R. and a Postgraduate Research Fellowship to C.J.J. from the Australian Wheat Research Council.

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