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Genetic analyses of rDNA spacer-length variation in barley

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Summary. The genetic mechanism controlling the inheritance of single and multiple spacer-length variant (slv) phenotypes in barley was investigated in six F_2 segregating populations. The results indicated that two independently assorting loci, each with co-dominant alleles, govern genetic variability for rDNA in barley regardless of the number of bands expressed by a given phenotype. The following chromosomal locations are proposed: sl variants 1, 4, 5, 6, and 7 on chromosome 7, and sl variants 7, 8, 9, 12, and 13 on chromosome 6; sl variant 7 is thus located on both of the chromosomes.

Key words: Spacer length variants – rDNA – Mendelian inheritance – *Hordeum spontaneum* – *Hordeum vulgare*

Introduction

The chromosomal locations of ribosomal DNA (rDNA) in many species have been determined by in situ hybridization of radioactively labelled RNA or DNA to denatured nucleic acid in cytological materials. In cultivated barley, *Hordeum vulgare* L., Appels et al. (1980) showed that rDNA is localized in the nucleolus organizer regions of two pairs of barley chromosomes. Metaphase preparations showed that the rDNA was almost entirely confined to chromosome regions immediately proximal to the secondary constriction. Their calculations indicated that approximately 1,200 repeat units occur on one chromosome and 600 repeat units on the other chromosome. More recently, genetic variation in the length of rDNA repeat units has been used to assign particular rDNA variants to individual chromosomes in wheat (Appels and Dvorak 1982; Flavell 1985; Flavell et al. 1986).

Ribosomal DNA or rDNA genes are organized as families of tandemly repeated genes that may comprise the nucleolar organizer regions of chromosomes. They are synthesized as a single precursor RNA that is processed into the mature 17S, 5.8S and 25S rRNA. Each repeat unit of rDNA contains a single rRNA transcription unit as well as an intergenic spacer (IGS) region that separates the transcription unit from the adjacent repeat unit. The IGS region of each repeat unit contains an array of tandemly repeated sequences, referred to as 'subrepeats', which are typically 100–300 base pairs (bp) in plants.

Gerlach and Bedbrook (1979) have constructed a physical map of rDNA repeats of *Eco*RI digests of barley and wheat; they defined two major repeat classes for rDNA in barley and one major and two minor classes of rDNA repeats in wheat. Within most species the length of the subrepeats varies by no more than a few base pairs, while the number of tandom copies of subrepeats within rDNA repeats is extremely variable.

Saghai-Maroof et al. (1984) studied spacer-length variation in ribosomal RNA gene clusters (rDNA) in cultivated barley (*Hordeum vulgare* L.) and its wild ancestor (*Hordeum spontaneum* L.). They reported that in total 17 rDNA spacer-length (sl) phenotypes, made up of 15 different rDNA sl variants (slvs), existed in barley. The 15 rDNA variants comprised a complete ladder in which each variant differed in length from its adjacent variant by approximately 115 base pairs (bp). Studies of 4 rDNA sl variants (4, 7, 8, 12) in an F_2 population showed that these variants were located at two unlinked loci, *Rrn1* and *Rrn2*, each with co-dominant alleles. Saghai-Maroof et al. also demon-

strated that *Rrn1* and *Rrn2* were located on chromosomes 6 and 7, respectively. Their results suggested that sl variants 1–7 were located on chromosome 7 and sl variants 8–15 were located on chromosome 6. In a later study Zhang et al. (1990) identified more sl variants, giving a total of 20 sl variants existing in the IGS region. The 20 sl variants are organized in two families, one comprised of a regularly complete 8-step ladder (slvs 1–7) 4740–5430 base pairs (bp) long in the nucleolar organizer region of chromosome 7 and the other a 12-step ladder (slvs 8–18) 5545–6695 bp long in the nucleolar organizer region of chromosome 6.

Among the 17 phenotypes observed, 2 had only 1 sl variant; 8 phenotypes had 2 sl variants; 5 phenotypes had 3 sl variants; and the remaining 2 phenotypes had 4 sl variants. These results raise several questions that need to be answered. First, will the two gene segregations observed for sl variants 4, 7, 8, and 12 hold for the other 11 sl variants observed. Second, how accurate is the observation that sl variants 1–7 are determined by *Rrn1* and sl variants 8–18 by *Rrn2*. The experiments I am about to describe provide some answers to the above questions.

Materials and methods

Genetic materials

The genetic analyses of rDNA sl variants were studied in six F_2 populations. Table 1 gives a description of the parents used to construct these six F_2 populations. Each cross was made to answer one or more of the questions discussed earlier. For example, PI 296892 × F_{8-10} was made in an attempt to understand that genetic mechanisms controlling single and triple sl variant phenotypes and to test the observation that sl variants 1–7 were located on chromosome 7 and sl variants 8–15 were located on chromosome 6. To test the degree of homogeneity among rDNA arrays, the cross between PI 296897 + PI 220523 was made.

Table 1. Genetic materials used in the study of rDNA spacer length variants

Genotype	Species	Classification	Sl variant number
PI 220523	*H. spontaneum*	Wild barley	7, 12, 13
PI 282628	*H. spontaneum*	Wild barley	6, 8
PI 296892	*H. spontaneum*	Wild barley	1, 5, 8
PI 296897	*H. spontaneum*	Wild barley	7, 8
PI 382604	*H. spontaneum*	Wild barley	5, 7
Atlas 68	*H. vulgare*	Cultivated barley	4, 12
Algerian	*H. vulgare*	Cultivated barley	7, 10
Pamella blue	*H. vulgare*	Cultivated barley	7, 9
F_{8-10}[a]	*H. vulgare*	Cultivated barley	7

[a] Experimental line selected from F_8 of barley composite cross II

DNA preparation

Total cellular DNA was isolated from individual seedlings as described by Saghai-Maroof et al. (1984). Freeze-dried tissue was powdered with a mechanical mill, dispersed in extraction buffer (50 m*M* TRIS pH 8.0, 0.7 *M* NaCl, 10 m*M* EDTA, 0.1% 2-mercaptoethanol), and incubated at 60 °C for 60 min. Chloroform/octanol, 24:1 (vol/vol) was added to form an emulsion that was centrifuged at 5,125 *g* for 10 min. The aqueous phase was removed, and the DNA was precipitated by adding a two-thirds volume of isopropanol. The DNA was removed and transferred to a tube containing 76% ethanol, 10 m*M* NH_4OAc, and 0.25 m*M* EDTA, and then dissolved in 1.5 ml 10 m*M* NH_4OAc and 0.25 m*M* EDTA.

Detection of rDNA variants

One microgram of DNA was digested to completion with 2 u *Sst*I for 16 h at 37 °C. Electrophoresis was in 1.1% agrose and 100 m*M* TRIS-acetate, 12.5 m*M* Na acetate, and 1 m*M* EDTA, pH 8.1, at 2 V/cm for 36 h. Under these conditions, 5-to 6-kb fragments move 11–14 cm from the origin, and barley rDNA variants can be resolved unambiguously. DNA was transferred from these gels to a biodyne filter membrane as described by Southern (1975). The filter was hybridized to [^{32}P]-labelled pTA71, a clone of a wheat rDNA repeat (Gerlach and Bedbrook 1979). Nick translation and hybridization were done as described by Rigby et al. (1977) and Maniatis et al. (1982).

Quantitative determination of phenotypes observed in the F_2 segregating population of the cross PI 296892 × F_{8-10} was made using a Pharmacia LKB Ultra Scan XL laser densitometer. The densitometer combines high resolution laser densitometry with that of area scanning. Identification of bands was based on migration distance, quantitation, and molecular weight. The background was adjusted precisely so that the most intense bands or zones could be analyzed accurately, and the best suited method for maximum precision in background subtraction was employed. The densitometer also employed two methods of curve integration: signal to background and Gaussian fit. The latter method was selected because it allows for a precise curve fitting procedure. A Gaussian-shaped curve was fitted to the absorbance curve, and the area under the peak was calculated as the area under the Gaussian curve to the limit of the background line.

Results and discussion

Inheritance of sl variants 6, 7, 9, 10

To study the inheritance of sl variants 6, 7, 9, and 10, in relation to that of sl variants 4, 7, 8 and 12 of previously known chromosomal location, three crosses were made. The first cross was between PI 282628 (slvs 6, 8) and 'Atlas 68' (slvs 4, 12), the second cross was between PI 296897 (slvs 7, 8) and 'Pamella blue' (slvs 7, 9), and the third cross was between 'Atlas 68' (slvs 4, 12) and 'Algerian' (slvs 7, 10). When a cross was made between PI 282628 × 'Atlas 68', the F_1 progeny from the cross showed four bands (slvs 4, 6, 8, 12). An F_2 population obtained by selfing a single F_1 hybrid plant gave the phenotypic classes shown in Table 2. In total, nine phenotypic classes were observed as follows: the two parental phenotypes (two-banded),

Table 2. Observed numbers in F_2 progenies and 'goodness of fit' to 1:2:1:2:4:2:1:2:1 ratio for rDNA sl variants in the cross PI 282628 × 'Atlas 68'

Genotype	Sl variant number	Observed number									χ^2 ($df = 8$)
		4,12	4,6,12	6,12	6,8,12	4,6,8,12	4,8,12	4,8	4,6,8	6,8	
PI 282628	6,8										
Atlas 68	4,12										
PI 82628 × Atlas 68 F2		6	9	2	5	19	11	5	5	5	6.13

Table 3. Observed numbers in F_2 progenies and 'goodness of fit' to 1:2:1 ratio for rDNA sl variants in the cross PI 296897 × 'Pamella blue'

Genotype	Sl variant number	Observed number			χ^2 ($df = 2$)
		7,8	7,8,9	7,9	
PI 296897	7, 8				
Pamella blue	7, 9				
PI 296897 × Pamella blue F_2		18	27	8	3.78

the F_1 phenotype (four-banded), two additional two-banded phenotypes and four three-banded phenotypes. This suggests that two independently assorted loci, each with co-dominant alleles, govern genetic variability for rDNA in this hybrid. A test of 'goodness of fit' to the expected 1:2:1:2:4:2:1:2:1 ratio, gave a $\chi^2_{[8]} = 6.13$, $0.50 < P < 0.75$.

Table 3 gives the segregation data obtained for the PI 296897 × 'Pamella blue' cross. The two parents appear to have a common allele at one locus and different alleles at the other locus. An F_1 hybrid plant from this cross showed three bands (slvs 7, 8, 9). An F_2 population obtained by selfing a single F_1 hybrid plant gave three phenotypic classes: the two parental two-banded phenotypes (slvs 7, 8; slvs 7, 9), and the F_1 phenotype (three-banded, slvs 7, 8, 9). A test of 'goodness of fit' to the expected 1:2:1 ratio, gave a $\chi^2_{[2]} = 3.78$, $0.50 < P > 0.10$.

Table 4 gives the phenotypic classes resulting from the cross between 'Atlas 68' × 'Algerian'. The phenotypic classes observed in the two parents, 'Atlas 68' and 'Algerian', were sl variants 4, 12 and 7, 10, respectively. All four bands appear in the F_1 hybrid. A total of nine phenotypes were observed in the F_2 plants, as follows: the two parental phenotypes (two-banded), the F_1 phenotype (four-banded), two additional two-banded phenotypes, and four three-banded phenotypes. Again, these results suggest that two independently assorted loci, each with codominant alleles, govern genetic variability for rDNA in this hybrid. A test of 'goodness of fit' to the expected ratio of 1:2:1:2:4:2:1:2:1 gave $\chi^2_{[8]} = 4.6$, $0.75 < P > 0.90$. It is quite clear from the results presented above that regardless of the spacer-length variants being studied, the end results are always the same, i.e., two independently inherited loci, each with co-dominant alleles, govern rDNA phenotypes in barley, and that all of the sl variants tested follow the same general Mendelian principles.

Inheritance of single and triple sl variant phenotypes

To understand the genetic mechanisms governing single and triple sl variant phenotypes, two crosses were made. The first cross made was between PI 296892

Table 4. Observed numbers in F_2 progenies and 'goodness of fit' to 1:2:1:2:4:2:1:2:1 ratio for rDNA sl variants in the cross 'Atlas 68 × 'Algerian'

Genotype	Sl variant number	Observed number									χ^2 ($df = 8$)
		7,10	4,7,10	4,10	7,10,12	4,7,10,12	4,10,12	7,12	4,7,12	4,12	
Atlas 28	4,12										
Algerian	7,10										
Atlas 68 × Algerian F2		4	6	3	11	20	9	5	10	3	4.6

Table 5. Observed numbers in F_2 progenies and 'goodness of fit' to 1:3:8:3:1 ratio for rDNA sl variants in the cross PI 296892 × F_{8-10}

Genotype	Sl variant number	Observed number					χ^2 ($df = 4$)
		1,5,8	1,5,7	1,5,7,8	7,8	7	
PI 296892	1, 5, 8						
F_{8-10}	7						
PI 296892 × $F_{8-10}F_2$		6	21	33	3	2	15.26**

** $P \leq 0.01$

(slvs 1, 5, 8) and F_{8-10} (slv 7), while the second cross was between PI 296897 (slvs 7, 8) and PI 220523 (slvs 7, 12, 13). The progenies of the first cross showed four bands. An F_2 population, obtained by selfing a single F_1 hybrid plant, gave the phenotypic classes given in Table 5 and Fig. 1. As can be seen from Table 5, five phenotypic classes were observed in this hybrid, as follows: the two parental phenotypes (three-banded, slvs 1, 5, 8; one-banded, slv 7), the F_1 phenotype (four-banded, slvs 1, 5, 7, 8), a three-banded nonparental phenotype, (slvs 1, 5, 7), and one two-banded phenotype (slvs 7, 8). Two models are possible. One is that sl variants 1, 5, 7 are determined by one locus, and sl variant 8 by another locus independently recombined with the first one. The other is that sl variant 7 is controlled by two loci (*Rrn1* and *Rrn2*). Assuming that the first model is correct, we would expect that the parental phenotype with sl variant 7 will be null at *Rrn2* and that six phenotypic classes would result as follows: two parental phenotypes

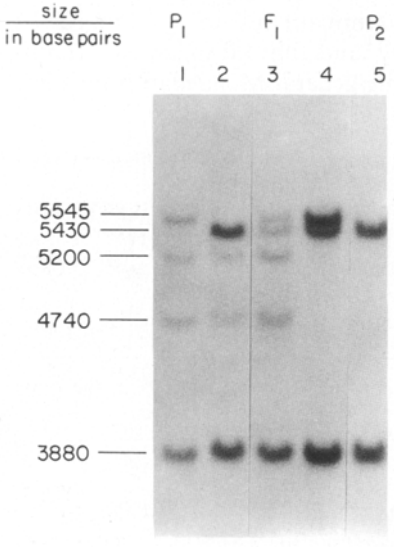

Fig. 1. Autoradiograph of Southern blot showing the five phenotypes observed in an F_2 population derived from the hybrid between PI 296892 (P_1) and F_{8-10} (P_2)

(three-banded, slvs 1, 5, 8; one-banded, slv 7), the F_1 phenotype (four-banded, slvs 1, 5, 7, 8), two two-banded phenotypes (slvs 7, 8; 1, 5), and a three-banded phenotype (slvs 1, 5, 7), with a 3:1:6:3:1:2 ratio, respectively. No phenotypic classes with sl variants 1 and 5 were observed. A test of 'goodness of fit' gave $\chi^2_{[5]} = 38.63$; $P < 0.01$. This suggests that two loci with co-dominant alleles govern genetic variability for rDNA in this hybrid. A test of 'goodness of fit' for the 1:3:8:3:1 ratio of observed to expected numbers, assuming independent inheritance, gave $\chi^2_{[4]} = 15.26$; $P < 0.01$. This expected ratio fits much better to the observed one. The significant χ^2 value is due primarily to deficiency in the phenotypic class with sl variants 7, 8 and excess in the phenotypic class with sl variants 1, 5, 7.

The above results allow the following deductions to be made: (1) the observed F_2 segregations are consistent with the hypothesis that two independently inherited loci, each with co-dominant alleles, govern rDNA phenotypes in this hybrid; (2) the single-banded phenotype with sl variant 7 is not a single-banded phenotype, instead it is a double-banded phenotype with the same sl variant 7; one sl variant 7 is controlled by *Rrn2* and the other sl variant 7 is controlled by *Rrn1*. Both sl variants 7 are of the same size, and when electrophoresed both migrate the same distance and appear as a single band. (3) In the triple-banded phenotype with sl variants 1, 5, and 8, sl variants 1 and 5 appear to be linked to chromosome 7 and sl variant 8 is linked to chromosome 6. To test the validity of these deductions, four phenotypic classes were progeny tested (three-banded, slvs, 1, 5, 8; three-banded, slvs 1, 5, 7; two-banded, slvs 7, 8; and one-banded, slv 7). The three-banded phenotype with sl variants 1, 5, 8 and the one-banded phenotype with sl variant 7 both bred true, while the three-banded phenotypes with sl variants 1, 5, 7 and the double-banded phenotype with sl variants 7, 8 each gave two genotypes when the intensity (copy number) of the critical bands was taken into consideration (see Table 6).

As to the second cross, PI 296897 contained sl variants 7, 8, while PI 220523 contained sl variants 7, 12,

Table 6. Densitometric scan of single plants representing the five phenotypes observed in the F_2 segregating population of PI 296892 × F_{8-10} cross[a]

Phenotype/genotype	Variants number	Size in base pairs	Peak height	Peak area	% of total
$P_1(1)$ 1 5 8 / 1 5 8	1	4740	0.125	0.45436	15.5
	5	5200	0.101	0.32280	11.0
	8	5545	0.160	0.70782	24.1
(2) 1 5 7 / 1 5 7 or 1 5 7 / 7 7	1	4740	0.109	0.60968	9.0
	5	5200	0.110	0.43664	6.4
	7	5430	0.529	2.69842	39.7
$F_1(3)$ 1 5 8 / 7 7 or 1 5 8 / 1 5 7	1	4740	0.169	0.83316	15.1
	5	5200	0.162	0.81242	14.7
	7	5430	0.177	0.71928	13.0
	8	5545	0.136	0.45128	8.2
(4) 7 8 / 7 8 or 7 8 / 7 7	7	5430	0.720	2.97000	23.5
	8	5545	0.859	4.41522	35.0
$P_2(5)$ 7 7 / 7 7	7	5430	0.559	2.97430	44.7

[a] See Fig. 1

Table 7. Observed numbers in F_2 progenies and 'goodness of fit' to 1:2:1 ratio for sl variants in the cross, PI 296897 × PI 220523

Genotype	Sl variant number	Observed number			χ^2 ($df = 2$)
		7,8	7,8,12,13	7,12,13	
PI 296897	7,8				
PI 220523	7,12,13				
PI 296897 × PI 220523 F_2		10	12	19	10.8**

** $P \leq 0.01$

Table 8. Observed numbers in F_2 progenies and 'goodness of fit' to 1:3:8:3:1 ratio for rDNA sl variants in the cross PI 296897 × PI 382604

Genotype	Sl variant number	Observed number					χ^2 ($df = 4$)
		5,8	5,7	5,7,8	7,8	7,7	
PI 296897	7,8						
PI 382604	5,7						
PI 296897 × PI 382604 F_2		0	16	30	10	2	5.03

13. The F_1 progeny from this cross showed four bands (slvs 7, 8, 12, 13). An F_2 population obtained by selfing a single F_1 hybrid plant gave the phenotypic classes given in Table 7 and Fig. 2. Only three phenotypic classes were observed in this hybrid, as follows: the two parental phenotypes (two-banded slvs 7, 8; three-banded, slvs 7, 12, 13) and the F_1 phenotype (four-banded, slvs 7, 8, 12, 13). This suggests that sl variants 12, 13 are allelic to sl variant 8. Assuming that sl variants 12 and 13 locate to chromosome 6 as sl

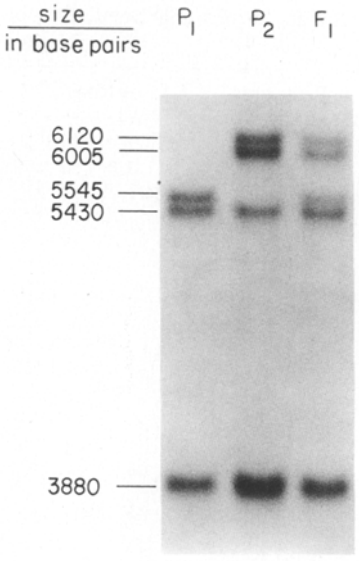

Fig. 2. Autoradiograph of Southern blot showing the three phenotypes observed in an F_2 population derived from the hybrid PI 296897 (P_1) and PI 220523 (P_2)

variant 8 does and that sl variant 7 locates to chromosome 7, a 1:2:1 ratio is expected for 7, 12, 13: 7, 8, 12, 13: 7, 8. A test of 'goodness of fit' of the observed ratio to the expected one gave $\chi^2_{[2]} = 10.8$; $P < 0.01$. The significant χ^2 value is due totally to deficiency in the heterozygous class with sl variants 7, 8, 12, 13 and excess in the parental class with sl variants 7, 12, 13, which suggest that the heterozygous phenotype is at a disadvantage relative to the parental phenotype with sl variants 7, 12, 13. This further supports the finding by Saghai-Maroof et al. (1984, 1990) and Zhang et al. (1990) that sl variants are under selection. It is concluded that (1) the genetic mechanisms governing single-and triple-banded sl variants follow the same Mendelian principles as the double-banded sl variants; (2) regardless of the number of sl variants expressed in a given individual, the inheritance of rDNA sl variants in barley is controlled by two independently inherited loci, each with co-dominant alleles, (3) deviation from the expected in the above crosses suggests that some sl variants are more advantageous in their transmission than others.

Quantitative analysis on sl variants' phenotypes

Since the number of repeating units of one rDNA gene is twice that of the other (1,200 versus 600) (Appels et al. 1980), it is expected that the variant copy numbers contributed by either *Rrn1* or *Rrn2* are not equal and are dependent of the number of variants at a particular locus, the chromosomal location, and the genotype of the individual being analyzed. Table 6 shows densitometric scans of the five phenotypes observed in the F_2 segregating population of PI 296892 × F_{8-10} cross. By carefully analyzing the results in Table 6, I deduced the following: the variant copy numbers contributed by either *Rrn1* or *Rrn2* are not equal; for example, in the parental type with sl variants 1, 5, 8, the number of copies of sl variants 1, 5 is equal to that of sl variant 8. It should be noted that genetic analysis indicated that sl variants 1, 5 are coded by *Rrn2* (chromosome 7) and sl variant 8 is coded by *Rrn1* (chromosome 6). On the other hand, for the phenotype with sl variant 7, 8, the number of copies contributed by *Rrn1* (slv 8) is significantly higher than the number of copies contributed by *Rrn2* (slv 7). In contrast, for the phenotype with sl variant 1, 5, 7, again the number of copies for both sl variant 1 and 5 is 40% of that observed for sl variant 7. This result can be explained as follows. This phenotype is possibly a mixture of two genotypes: one genotype is homozygous for sl variants 1, 5 (*Rrn2*) and sl variant 7 (*Rrn1*), and the second genotype is heterozygous for sl variants 1, 5, 7 (*Rrn2*) and homozygous for sl variant 7 (*Rrn1*). In effect, three doses of variant 7 would be present versus one dose for each of variant 1 and 5. It should be noted that two of these doses (slv 7) would be contributed by *Rrn1* and the third dose would be contributed by *Rrn2*. Progeny testing of the two phenotypes with sl variants 1, 5, 7 and 7, 8 segregated to two genotypes as predicted above.

On the chromosomal location of sl variant 7 alleles

Saghai-Maroof et al. (1984) suggested that sl variants 1–7 were located on chromosome 7 and that sl variants 8–15 were located on chromosome 6. Two crosses were used to test these deductions. The first cross was between PI 296897 (slvs 7, 8) and PI 382604 (slvs 5, 7), while the second cross was between PI 296892 (slvs 1, 5, 8) and F_{8-10} (slv 7). The results are given in Tables 8 and 5, respectively. The results given in Table 5 were discussed earlier. When PI 296897 was crossed to PI 382604, the F_1 progenies from this cross showed three bands (slvs 5, 7, 8). An F_2 population obtained by selfing a single F_1 hybrid plant gave the phenotypic classes shown in Table 8. Four phenotypic classes were observed as follows: the two parental phenotypes (two-banded), the F_1 phenotype (three-banded, slvs 5, 7, 8), and one single-banded phenotype. If, on the other hand, the two parents differed at two loci, more than three phenotypic classes would be observed in the F_2s. This was shown to be the case (Table 8). It has been established (Saghai-Maroof et al. 1984) that sl variants 7, 8 of PI 296897 are located on chromosome 7 and 6, respectively. Assuming that sl variant 7 of PI 382604 is allelic to sl variant 8 of PI 296897 and that sl variant 5 of PI 382604 is allelic

to sl variant 7 of PI 296897, I can expect a 1:3:8:3:1 ratio for sl variants 5,8: 5,7: 5,7,8:7,8:7, assuming independent inheritance of the loci. A test of 'goodness of fit' of the observed ratio to the expected gave $\chi^2_{[4]} = 5.03$, $0.25 < P > 0.10$. These results suggest that sl variants 5 and 7 of PI 382604 are located on chromosomes 7 and 6, respectively.

The results presented earlier for the PI 296892 × F_{8-10} cross clearly show that the single-banded phenotype with sl variant 7 is actually a two-banded phenotype with one sl variant 7 located on chromosome 7 and the other sl variant 7 on chromosome 6 or 7, and that genetic analysis is the only means of identifying their allelic relationship. Now it appears likely that sl variants 1–7 and sl variants 7–18 are located on chromosome 7 and 6, respectively. I assume that the origin of the two sl variants 7s on these chromosomes is different.

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