Genetic regulation of trypsin inhibitory activity in soybean flour

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Abstract: Many biochemical and molecular details are available for soybean seed proteinase inhibitors, but little is known about the quantitative regulation of structural genes. In order to fill this gap, a complete set of diallel crosses was made between inbred lines characterised by the same inhibitor array coupled with a different inhibitor content in the raw flour. The Hayman analysis revealed the presence of different regulatory elements in the parental lines, giving rise to both additive and dominance variation. Dominant alleles were found to decrease the trypsin inhibitory activity (TI activity) shown by the raw flours and appeared to have higher frequencies than recessive alleles. Significant maternal effects were also detected, particularly in crosses of low-TI activity female × high-TI activity male; maternal effects strengthened the role of the regulatory genes transferred by the female parent to the hybrid. Data fitted the simple additive–dominance model with genes independent in both action and distribution. Narrow and broad heritability values were 54% and 82% respectively, thus indicating the feasibility of lowering TI activity through selection.

Keywords: Kunitz inhibitor; Bowman–Birk inhibitors; expression; regulation; soybean; diallel analysis

INTRODUCTION

The seed of the soybean contains several proteinase inhibitors and other antinutritional factors which preclude the employment of raw soybean meal in human and livestock nutrition.[1–3] Owing to their nutritional significance and the high processing costs involved in their inactivation, soybean proteinase inhibitors are still being intensively studied.

The Kunitz inhibitor (or SBTI-A_2 protein)[4] is a water-soluble, 21 kDa molecule extremely active against trypsin. Three electrophoretic forms have been identified by screening the USDA soybean germplasm collection;[5,6] these variants are all codified at locus *Ti* through a system of multiple codominant alleles (Ti^a, Ti^b and Ti^c); *Ti* alleles show an uneven geographical distribution[7,8] and code for peptides with a markedly different inhibitory effect against bovine trypsin.[9] *Ti* transcription is clearly tissue-specific; in mid-ripening embryos, the Kunitz inhibitor mRNA accounts for 4% of the total mRNA,[10] whereas in other organs such as leaves, stems or roots, transcription is 10^3 times lower than in developing embryos. In dormant seed, no mRNA for this factor has been detected.

The Bowman–Birk-type inhibitors[11] are double-headed proteins which are related to each other by one or more features, eg homology in the coding sequence, molecular weight, number of glycine or cysteine residues, isoelectric point, inhibitory spectrum and/or cross-reaction with different antibodies. To date, the most complete classification has been given by Tan-Wilson *et al*,[12] who reported that there are four different groups of Bowman–Birk inhibitors and this number probably corresponds to the minimal number of genes involved in their synthesis. In fact, many inhibitors are thought to derive from active precursors through terminal cleavage.[13] A seed-specific expression for the classic Bowman–Birk inhibitor has been recognised by several authors;[14,15] Hammond *et al*[14] also found that the mRNA for this inhibitor accumulates at the same rate and developmental stages as the Kunitz trypsin inhibitor mRNA. On the other hand, soybean seeds generally have more Kunitz than Bowman–Birk inhibitor;[16] a possible explanation for this could be the presence of regulatory mechanisms based on factors other than mRNA levels in the developing seed. Another possible explanation could be related to the fact that the expression levels of the two genes actually differ in the majority of genotypes.

Marchetti *et al*[17] observed significant differences in the antitryptic activities shown by seed samples collected from different positions of the main stem. They also noted that the variation in antitryptic activity along the main stem of Williams 82 was very similar to that found in its near-isogenic line L81-4590 (which is recessive at locus *Ti* and thus unable to synthesise the Kunitz factor[18]); they therefore sug-

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gested that the topological effect is related to the differential synthesis of inhibitors other than the Kunitz inhibitor.

In order to verify the presence of regulatory mechanisms in the biosynthesis of trypsin inhibitors and to elucidate the type of genetic control, a diallel set of crosses was produced using parental lines carrying the same structural genes. The present paper reports on the results of the diallel analysis carried out on data regarding the trypsin inhibitory activity (TI activity) of hybrid seed flour.

EXPERIMENTAL

Six accessions of soybean (*Glycine max* Merr) from the USDA germplasm collection were used in the experiment: BSR 301, Elf, Gnome 85, Pella 86, Richland and Williams 82. These cultivars belong to the maturity groups II or III and their flours are characterised by different levels of TI activity. In order to check the inhibitor composition of each genotype, a biochemical investigation based on affinity chromatography, anion exchange chromatography, reverse phase chromatography and trypsin inhibition assay was carried out. As the Kunitz trypsin inhibitor in all the cultivars tested is encoded by the Ti^a allele, the investigation was mainly concerned with the identification of the array of Bowman–Birk-type inhibitors.

Inhibitor extraction and purification

A seed sample (10 g) from each parental inbred was ground in an analytical mill (Retsch model ZM1, 1 mm screen), and 100 mg of the resulting flour was extracted in 10 ml Tris-HCl (10 mM, pH 8.0) for 30 min at 4 °C. Following centrifugation at $5000 \times g$ for 15 min, the supernatant was collected and filtered through a 0.45 μm sieve; to reduce non-specific binding, NaCl was added to 5 ml of supernatant to give a final concentration of 0.5 M. Trypsin inhibitors were purified from extracts by affinity chromatography on trypsin-conjugated agarose (Sigma Chemical Co); 1.5 ml of gel was obtained by pouring 3 ml of cross-linked beaded agarose suspension in 10 mM acetic acid into a Poly-Prep Chromatography Column (Bio-Rad Laboratories). The resin was washed with 150 ml Tris-HCl (10 mM, pH 8.0) containing 0.5 M NaCl, loaded with sample and gently rotated for 20 min at room temperature. The reaction fluid was passed through the column, collected and analysed for TI activity as the initial supernatant. The column was then washed with 50 ml Tris-HCl (10 mM, pH 8.0) to remove non-specifically bound compounds. Inhibitors were eluted with 8 ml glycine-HCl buffer (0.1 M, pH 2.6),[19,20] and 1 ml fractions were collected and analysed separately. All fractions showing TI activity were pooled and concentrated approximately 25-fold in a 50 ml Amicon diafiltration cell on a YM 1 (1 kDa) membrane (Amicon Grace Co). The column was washed with 50 ml Tris-HCl (10 mM, pH 8.0) containing 0.5 M NaCl, and the absence of inhibitors was checked in the first 1 ml fraction. The protein composition of the active fractions was checked by SDS-PAGE using a concentration of polyacrylamide ranging from 16 to 20% in Tris-glycine[21] or Tris-tricine[22] buffer system; polypeptide molecular weight standards from Bio-Rad and purified Kunitz trypsin inhibitor and Bowman–Birk inhibitor (Sigma Chemical Co) were used as electrophoretic markers.

Anion exchange chromatography

Concentrated samples were analysed by HPLC using a Jasco 875-UV apparatus equipped with an anionic exchange column HRLC^R MA7Q 50 mm × 7.8 mm (Bio-Rad Laboratories) equilibrated with 10 mM Tris-HCl, pH 8.5 (buffer A). Sample (200 μl) was injected and elution was performed at a flow rate of 5 ml min^{-1} using 0.125 M NaCl solution in 10 mM Tris-HCl, pH 5.1 (buffer B). After injection, the concentration of buffer B was raised to 25% in 1 min; protein separation was obtained in a linear gradient of buffer B (25–100% in 10 min). Proteins were detected by recording the absorbance at 280 nm. Purified Kunitz trypsin inhibitor and Bowman–Birk inhibitor (Sigma Chemical Co) were used as standards.

Reverse phase chromatography

Concentrated samples were also analysed by reverse phase HPLC on a C18 column (Perkin-Elmer) using a Jasco 875-UV apparatus. Trifluoroacetic acid was diluted in doubly distilled water (solution A) and in acetonitrile (solution B) to a final concentration of 0.1% v/v; the column was equilibrated with 90% solution A and 10% solution B. Sample (20 μl) was injected and elution was performed at a flow rate of 1.5 ml min^{-1} using the following discontinuous gradient of solution A and B: 80% A and 20% B in 2 min; 30% A and 70% B in 30 min; 5% A and 95% B in 5 min. Proteins were detected by recording the absorbance at 280 nm. Purified Kunitz trypsin inhibitor and Bowman–Birk inhibitor were used as standards.

Trypsin inhibition assay

This was performed as described by Smith *et al*,[23] with some modifications.[24] The inhibitory activity was determined on a micro-ELISA plate (Dynatech Instruments Inc) using the following incubation medium: 20 μl flour extract in Tris-HCl (10 mM, pH 8.0; for maximum trypsin activity, 20 μl extraction buffer was used), 20 μl 1 mM HCl containing 2 μg trypsin (bovine pancreas, crystallised twice; Sigma Chemical Co), 160 μl 1 mM BAPNA (Na-benzoyl-DL-arginine-*p*-nitroanilide; Sigma Chemical Co) in 50 mM Tris-HCl containing 20 mM $CaCl_2$, pH 8.2. After 20 min incubation at 20 °C, the reaction was stopped by adding 50 μl acetic acid (30% v/v in water), and the absorbance at 405 nm was read using a micro-ELISA auto-reader (Dynatech Instruments Inc).

A linear relationship between flour concentration in the extract and trypsin activity was found in all cases.

Different dilutions of the raw flour extracts in Tris-HCl (10 mM, pH 8.0) were made in order to reduce trypsin activity to 40–60% of the maximum. Three independent analyses were carried out for each flour; the antitryptic activity shown by a sample was expressed in terms of mg trypsin inhibited g^{-1} flour.

Hybrid seed production for diallel analysis

Plants were grown on a dystric ferralic cambisol at Udine, north-east Italy. Seeds were planted on 20 May 1994 with a 0.75 m row spacing at a rate of 20 seeds per metre. Plots consisted of single-plant progenies allocated in three bordered 4 m rows; more than 20 genetic markers of morphological type were considered to exclude the presence of off-types in the plots. Crosses were made according to Johnson and Bernard[25] on female plants of the same developmental stage.[26] In order to further minimise epigenetic variation,[17,24] crosses were carried out at the 13th node of the main stem and all within 1 week. Selfed seed was produced at the same node and under the same conditions; in particular, since the crossing technique involves the removal of all flowers except the one used for crossing, only one flower was left at the 13th node even in the case of selfing. At physiological maturity, pods were harvested and manually threshed; there was no difference between crossing and selfing as to pod set, mean seed number per pod or mean seed weight. The seed was dried to constant weight in a ventilated oven at 35 °C and stored at 2 °C. Just before analysis, three replicates of 10 seeds each were formed for each diallel entry; each replicate was analysed in duplicate, but since the difference between the two values was trivial (less than 1%), only the first value was used.

Statistical analysis

Data were submitted to analysis of variance after checking (i) the normality of the distribution of data by means of the Kolmogorov–Smirnov test, (ii) the homogeneity of variances with Bartlett's formula and (iii) the lack of any correlation between variance (or standard deviation) and mean. The diallel table was analysed as described by Hayman;[27] to verify whether or not an additive–dominance model involving independently distributed genes could provide a realistic picture of the data, the variance/covariance analysis developed by Jinks[28] was used.

RESULTS AND DISCUSSION

As found in other experiments,[29–31] affinity chromatography on trypsin-conjugated agarose was highly effective in isolating trypsin inhibitors from the bulk of soluble seed proteins; in no instance were the unbound fractions displaying residual TI activity. The first 1 ml fraction from column washing was also completely inactive.

On SDS-PAGE, the six soybean inbreds appeared to share the same electrophoretic pattern (Fig 1); as

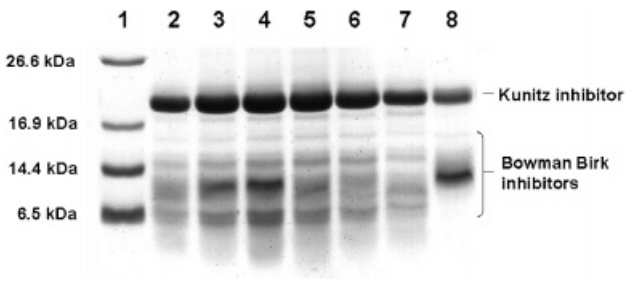

Figure 1. SDS-PAGE on 20% polyacrylamide according to Laemmli.[21] Lane 1: polypeptide molecular weight standards (Bio-Rad). Lanes 2–7: active fractions purified by affinity chromatography on trypsin-conjugated agarose from flours of Gnome 85, BSR 301, Elf, Pella 86, Richland and Williams 82 (5 μg protein per lane). Lane 8: 5 μg purified Kunitz trypsin inhibitor (Sigma Chemical Co) combined with an equal amount of Bowman–Birk inhibitor (Sigma Chemical Co).

expected, all purified samples contained the Kunitz trypsin inhibitor and several bands with lower molecular weight corresponding to different Bowman–Birk-type inhibitors. With Tris-tricine, protein separation was satisfactory but bands were not sharply defined; a better resolution was achieved with 20% polyacrylamide and Tris-glycine as buffer system.

When soybean inbreds were analysed by anion exchange chromatography, different peak widths were noted but the chromatographic profile was the same (Fig 2) in all cases. Through a comparison of the retention times, the widest peak was found to correspond to the Kunitz trypsin inhibitor; Bowman–Birk inhibitors present in all samples were characterised by retention times lower than 10 min; proteins found in the Bowman–Birk inhibitor preparation from Sigma were also present in the sample profile at their expected positions (data not shown).

It must be pointed out that the profile in the Bowman–Birk section of the chromatogram appeared to be composed of 10 different proteins, which is the recognised complement of Bowman–Birk isoinhibitors in soybean.[32]

Soybean samples also appeared similar when analysed by reverse phase chromatography, in that the number of peaks and their retention times were always the same (Fig 3); in particular, 10 different peaks were noted, the one with a 20.3 min retention time being the Kunitz trypsin inhibitor. As with anion exchange chromatography, Bowman–Birk-type inhibitors appeared distributed in the first part of the chromatogram; in all cases, the two major components were peak 6 and peak 2, characterised by a retention time of 11.0 and 9.2 min respectively (Fig 3).

Despite the similarity observed with chromatographic methods capable of detecting changes in the net charge of the proteins and their hydrophobicity, TI activities shown by the raw flours varied significantly; in agreement with previous observations,[24] a particularly low value was obtained for cvs Richland and Pella 86 (Table 1). Differences between soybean genotypes have been documented previously,[17,24,33,34] but to the authors' knowledge, no such difference has ever been

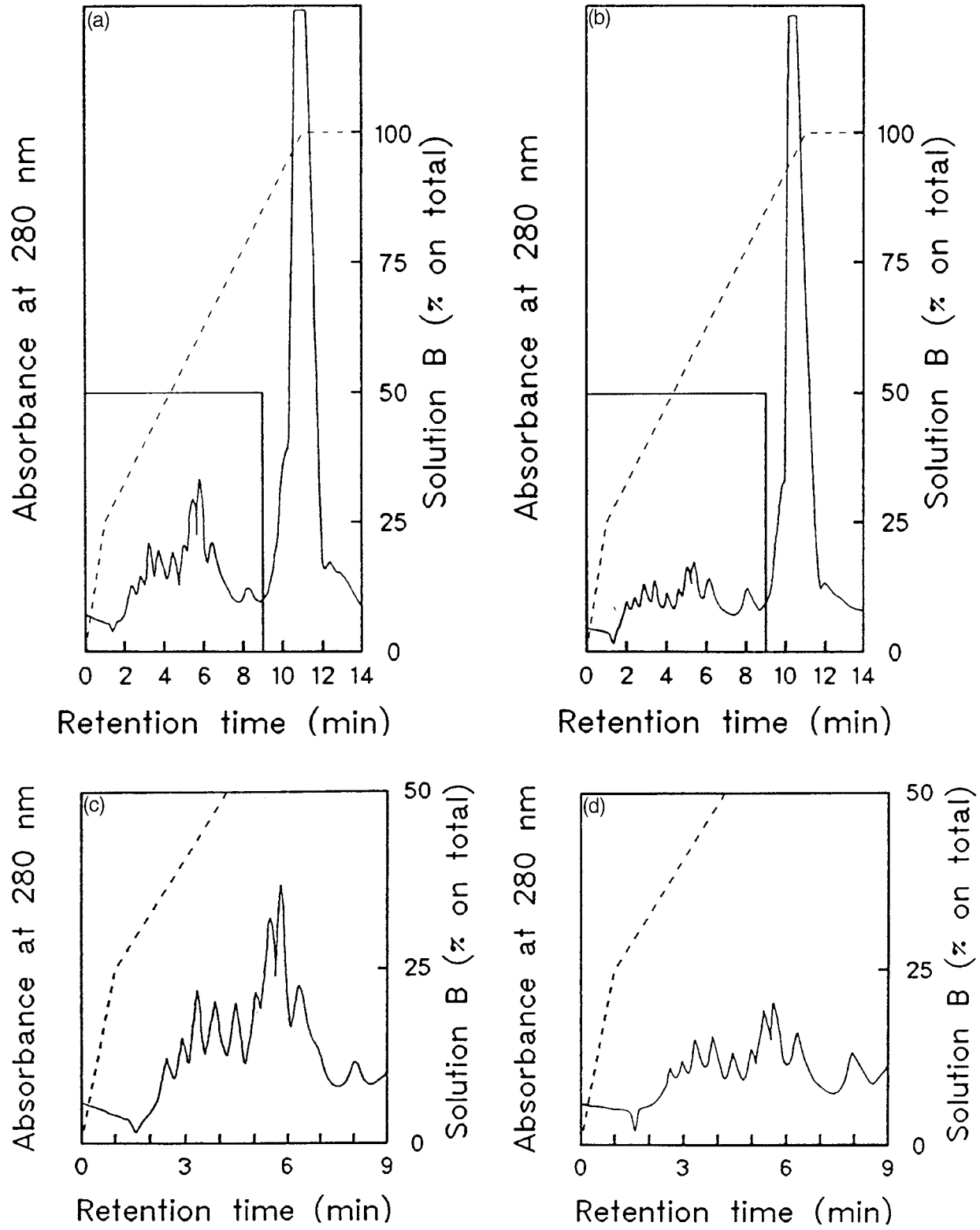

Figure 2. Examples of profiles from anion exchange chromatography carried out on an HRLC® MA7Q column (Bio-Rad). Samples consisted of active fractions purified by affinity chromatography on trypsin-conjugated agarose. Profiles of (a) extract from flour of BSR 301 and (b) extract from flour of Pella 86. Windows of (a) and (b) concerning the Bowman–Birk section of the profile are shown in (c) and (d) respectively.

demonstrated for a range of cvs producing the same array of proteinase inhibitors.

The results of the Hayman[27] analysis of variance are presented in Table 2. The following item effects were examined: (a) additive genetic variation; (b) dominance variation; (b_1) mean dominance deviation of the F_1s from their mid-parental values; (b_2) mean dominance deviation of the F_1s from their mid-parental values within each array over arrays; (b_3) dominance deviations unique to each F_1; (c) variation due to maternal effects; and (d) variation in reciprocal crosses not attributable to (c).

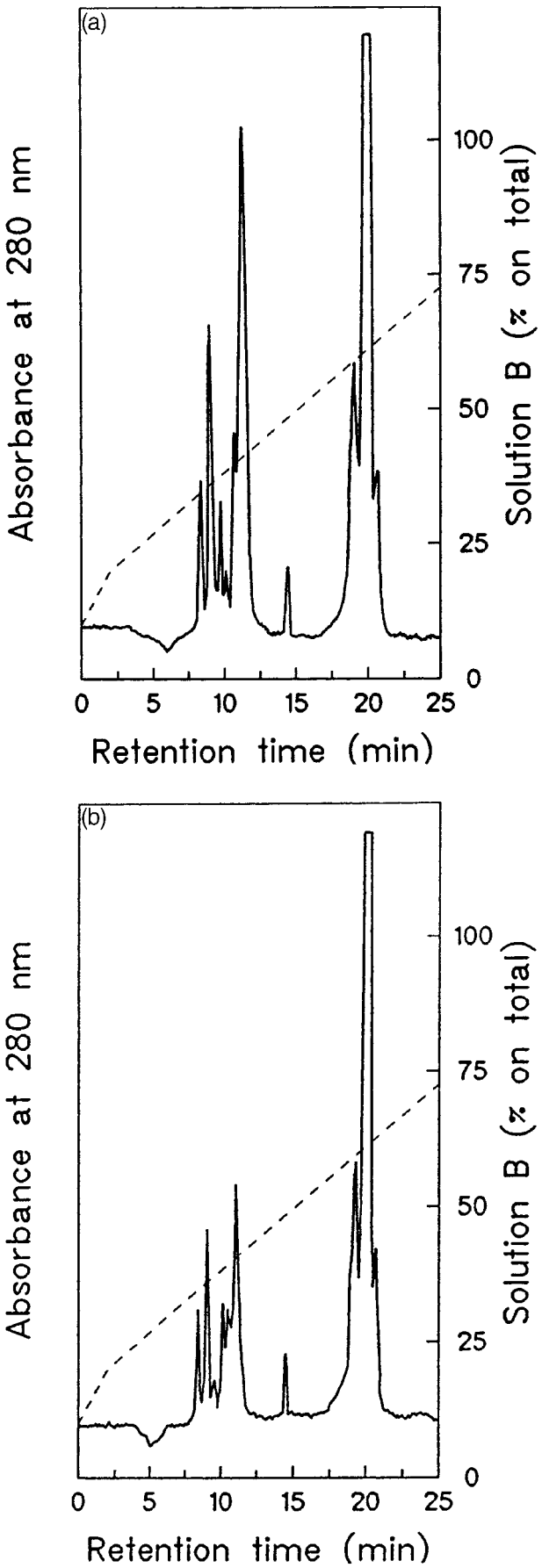

Figure 3. Examples of profiles from reverse phase chromatography carried out on a C18 column. Samples consisted of active fractions purified by affinity chromatography on trypsin-conjugated agarose. Profiles of (a) extract from flour of BSR 301 and (b) extract from flour of Pella 86.

All items were significant or highly significant when tested against their own interaction. Therefore, it can be concluded that the differences found between the parental lines were due to the presence of different regulatory elements, giving rise to both additive and dominance variation. As indicated by the significance of (b_1), the TI activity shown by hybrid seed statistically differed from the mid-parental value; reference to Table 1 shows that F_1s were very frequently characterised by lower antitryptic activities. In particular, 26 of 30 hybrids (87%) displayed a lower TI activity than that of the parent with lower TI activity. In all cases but one, exceptions to this rule involved crosses between a high-TI activity female and a low-TI activity male; it should be pointed out that hybrids derived from such combinations tended to be similar to the female parent. Maternal effects were even more evident in crosses of low-TI activity female $\times$ high-TI activity male; in this case, maternal effects always appeared to support the action of the regulatory genes transferred by the female parent to the hybrid. It can therefore be stated that the TI activity value displayed by the hybrid seed flour is partly dependent on the genetic constitution of the mother plant and that there could be some regulatory pathway followed by both hybrid embryo and maternal tissue. However, as soybean serine proteinase inhibitors often share the same inhibitory spectrum, it was not possible to deduce which structural genes were most involved in the regulatory mechanism on the basis of a simple enzymatic assay.

The adequacy of the additive–dominance model with genes independent in action and distribution was confirmed by testing the relationship between the variance (V_r) and parent–offspring covariance (W_r); since $W_r - V_r$ was constant over arrays whereas $W_r + V_r$ was not (Table 3), the presence of independent genes with dominant effects could be demonstrated. Results of the multiple regression analysis of W_r on V_r were also in agreement with the hypothesis of a simple additive–dominance model (Table 4). Furthermore, when W_r was regressed on V_r, no significant deviation from linearity was observed and the regression coefficients b and a were not significantly different from one and zero respectively (Fig 4). Therefore it might be assumed that dominance is complete and that dominant and recessive genes are independently distributed in the parental inbreds. As expected on the basis of phenotypic expression, Richland and Pella 86 appeared to possess the highest number of dominant alleles, whilst Elf had the most recessive ones.

After estimating the components of variation D, H_1, H_2, F and E, a high dominance ratio (0.981) and the presence of unequal allele frequencies (mean value of uv over all loci = 0.222) were revealed. Since many of the soybean inbreds used in this experiment are characterised by a relatively low TI activity (in comparison with most other accessions in the USDA germplasm collection[24]) and since dominant alleles generally have a decreasing effect on TI activity, a

Table 1. Average trypsin inhibitory activity (mg trypsin inhibited g^{-1} flour) ± standard error of the mean in the 6 × 6 diallel set of crosses

	Male					
Female	*Elf*	*Williams 82*	*Gnome 85*	*BSR 301*	*Pella 86*	*Richland*
Elf	34.1±1.9	27.7±0.7	26.8±0.8	24.8±0.5	28.5±1.1	24.8±0.3
Williams 82	28.6±0.4	29.8±1.2	29.7±0.2	25.1±0.5	28.0±0.3	27.8±0.7
Gnome 85	30.0±0.6	25.6±0.8	28.9±0.6	25.9±0.2	27.0±0.3	24.9±1.2
BSR 301	26.8±0.5	25.0±1.7	25.8±0.5	27.3±0.7	23.0±0.4	22.7±0.6
Pella 86	23.2±1.1	24.2±1.4	18.2±0.9	23.7±0.6	24.2±1.3	23.5±0.9
Richland	21.0±0.5	19.6±0.3	21.6±0.9	19.8±0.7	23.3±0.6	24.5±0.2

Table 2. Mean squares (MS), degrees of freedom (DF) and significance of the items in the Hayman analysis of variance

Item	*MS*	*DF*	*P*[a]
a	28.09	5	<0.01
b	5.16	15	<0.01
b_1	44.92	1	<0.05
b_2	4.24	5	<0.05
b_3	2.45	9	<0.01
c	15.44	5	<0.01
d	3.18	10	<0.01
Pooled B[b]	0.49	70	
B × a	0.93	10	
B × b	0.29	30	
B × b_1	0.02	2	
B × b_2	0.12	10	
B × b_3	0.42	18	
B × c	0.99	10	
B × d	0.32	20	

[a] Each item tested against its own block interaction.
[b] Block interactions.
(a) Additive genetic variation; (b) dominance variation; (b_1) mean dominance deviation of the F_1s from their mid-parental values; (b_2) mean dominance deviation of the F_1s from their mid-parental values within each array over arrays; (b_3) dominance deviation unique to each F_1s; (c) variation due to maternal effects; (d) variation in reciprocal crosses not attributable to (c).

Table 3. $W_r + V_r$ and $W_r - V_r$ analysed for trypsin inhibitory activity

Item	*DF*	*MS*	*P*
($W_r + V_r$) Array differences	5	21.52	<0.01
($W_r + V_r$) Block differences	12	2.08	
($W_r - V_r$) Array differences	5	0.52	NS
($W_r - V_r$) Block differences	12	0.25	

Table 4. Joint regression analysis of W_r on V_r

Item	*SS*	*DF*	*MS*	*P*
Total	44.10	15		
Regression	34.71	3		
Joint regression	34.08	1	34.08	<0.01
Heterogeneity	0.62	2	0.31	NS
Remainder	9.39	12	0.78	

prevalence of dominant alleles should be expected. This expectation was confirmed by the positive value of *F*.

As far as the application of these findings to conventional breeding is concerned, it should be pointed out that major advancements in lowering TI activity of raw soybeans can currently be achieved by crossing a *Ti* line with a strain or cultivar lacking the Kunitz trypsin inhibitor (*ti*). However, the simple removal of this inhibitor from seed without any intervention upon the Bowman–Birk inhibitor family, although allowing a reduction of the processing costs,[35] does not appear to solve the problem of direct livestock nutrition.[36] Unfortunately, despite extensive surveys, nulls for the classic Bowman–Birk inhibitor were not found in *G max* or *G soja*,[37] but only in perennial species for which problems of crossability or progeny fertility occur. Apparently, nulls for the C-II inhibitor (another important member of the Bowman–Birk inhibitor family) are also difficult to find; all *C-II* alleles described so far differ only at positions where degeneration of the code gives synonym triplets. The evidence presented in this paper indicates that significant variability can be observed for a range of regulatory elements which collectively are as important as the presence/absence of a Ti^a allele in the corresponding locus. Narrow and broad heritability for TI activity raised by the bulk of regulatory elements

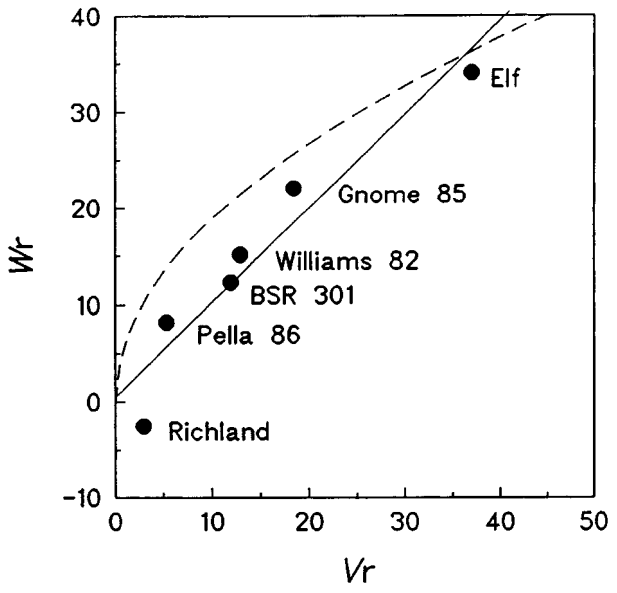

Figure 4. Relationship between W_r and V_r for TI activity.

was 53.8% and 82.0% respectively; these figures suggest that selection for a low TI activity in soybean flour should not be particularly difficult, even in the presence of a complete array of trypsin inhibitors and even when a subset of low-TI activity soybean inbreds is chosen as the starting material.

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