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Inheritance of qualitative and quantitative trypsin inhibitor variants in *Pisum*

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Abstract A trypsin inhibitor locus (*Tri*) has been mapped close to *Vc-2* on *Pisum* (pea) linkage group 5 using recombinant inbred lines derived from crosses of genotypes showing qualitative variation in seed trypsin inhibitors. F_2 seed populations derived from crosses between lines showing qualitative variation in trypsin inhibitors as well as quantitative variation in inhibitor activity showed an association between the segregation of the structural variation and relative activity levels. Clones complementary to *Pisum* trypsin inhibitor mRNA were used in hybridization analyses which showed that the segregation of protein polymorphisms reflected directly the segregation of polymorphisms associated with the structural genes.

Key words Genetic variation · *Pisum* · Trypsin inhibitor

Introduction

Seed trypsin inhibitors (TI) are considered to be important in determining the quality of legume seeds and are classified as antinutritional factors by the animal feeding industry. As such, quantitative variation in trypsin inhibitor activity (TIA) and its possible correlation with genetic and environmental factors have received attention in recent years (Savage 1989; Leterme et al. 1992; Grosjean et al. 1993). Pea (*Pisum sativum*) breeding programmes aimed at the animal feed industry have as one of their objectives the selection of lines having low levels of seed TI. Indeed, in France, there are price penalties involved in the sale of peas having more than a given (arbitrary) level of TI. Although environmental variation in TIA has been noted by others, we have shown previously that pea lines containing high or low levels of TI were consistently high or low, respectively, in a number of environments; these data suggested a strong genetic basis for the character (Domoney and Welham 1992).

Besides their interest to plant breeders as antinutritional factors, TI also attract considerable interest as protective agents against insect attack (Hilder et al. 1990; Johnson et al. 1989), and their involvement in wound responses in many species is currently receiving much attention (for review, see Ryan 1990). Furthermore, although their antinutritional role in the irreversible inhibition of digestive enzymes is well documented, protease inhibitors may also have a positive nutritional significance, due to their enriched content of sulphur-containing amino acids relative to the majority of seed proteins.

Information on the inheritance of TI and the numbers of genetic loci involved will be of direct relevance to pea breeding programmes. Several TI can be identified in any one genotype of pea. We have purified and characterized pea seed TI and shown that the isolated members are closely related to each other and are homologous to the Bowman-Birk class of inhibitors (Domoney et al. 1993a,b). However, it is not clear from the N-terminal sequences determined whether the variant polypeptides reflect the existence of multiple genes or whether post-translational modifications result in polypeptide polymorphism. In the investigation reported in this paper, we examined the genetics of TI variation and investigated the relationship between protein and gene polymorphisms.

Materials and methods

Plant material

The *Pisum* lines used were from the John Innes germ plasm collection. The recombinant inbred lines have been described previously (Ellis et al. 1992). Reciprocal crosses between selected lines were established and F_2 seed collected from plants grown in the greenhouse.

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Analysis of trypsin inhibitors and trypsin inhibitor activity

Individual F_2 seeds or bulked seeds from individual recombinant inbred lines (F_9 or F_{10} generation) were ground finely using a ball-mill. Samples of meal from F_2 seeds were extracted in 0.01 *M* NaOH, and quantitative TI assays were performed as described by Domoney and Welham (1992). TI units (TIU) were determined per milligram dry meal extracted, where 1 TIU gives a reduction in A_{410nm} of 0.01 relative to control reactions (Kakade et al. 1974). For gel analyses, meal was extracted in ten volumes of 0.03% (v/v) HCl, and the extracts dialysed against water and freeze-dried. Samples were analysed on non-denaturing gels, which were stained for trypsin activity as described previously (Domoney et al. 1993a). Variant patterns were scored and the data for the recombinant inbred lines analysed for association with previously scored characters (see Ellis et al. 1992). The F_2 data were analysed by chi-square test, and the recombination fraction was calculated according to Stevens (1940).

Analysis of DNA

Pea TI clones were obtained from cDNA libraries constructed using poly(A)-containing RNA isolated from embryos at late stages of development. Double-stranded cDNA was cloned into the *Sma*I site of pUC19. Libraries were screened using redundant oligonucleotide probes corresponding to portions of the N-terminal sequences determined for pea TI polypeptides (Domoney et al. 1993a,b). Hybridization was in 6 × SSC, 4 × Denhardts at 40°C. Filters were washed twice for 30 min in 6 × SSC, 0.1% SDS at 30°C. A relationship between positive clones and TI polypeptides was confirmed by DNA sequence analyses. A TI cDNA clone, pTI4-41, encompassing the entire mature protein-coding and 3′-untranslated sequences plus part of the 5′-leader sequence (Domoney and Welham, manuscript in preparation) was used for the genomic DNA hybridization analyses. The insert was removed by restriction with *Eco*RI and *Pst*I and subsequently labelled using α-[^{32}P] dCTP and an oligolabelling kit (Pharmacia).

Pea genomic DNA was prepared and restricted, and blots analysed by hybridization to labelled insert as described previously (Domoney et al. 1986). Filters were washed in 0.5 × SSC, 0.1% SDS at 65°C (TI hybridization) or 50°C (vicilin hybridization) and exposed to pre-flashed film at –70°C.

Results

Mapping qualitative TI variants

The analysis of seed from a number of pea lines on non-denaturing gels showed that several variant TI patterns were evident, with between 2 and 7 TI being distinguishable in any one genotype (Fig. 1). Quantitative differences in most of the TI were evident, and these patterns were consistent between seeds and between extracts for a particular genotype. When larger amounts of protein than shown in Fig. 1 were analysed, TI which appeared to be absent in particular genotypes could then be detected at a low level. The exception to this was the TI of greatest mobility, which could not be detected in some genotypes even with high protein loadings. Analysis of F_9 seeds from 65 recombinant inbred lines derived from the cross between JI 281 and JI 399 showed that 64 of these had either the JI 281 or the JI 399 pattern; that is, the presence of the TI of greatest mobility was always associated with a relative reduction in the amounts of the 3 TI of least mobility (Fig. 1). (The pattern of the remaining line was consistent with that of a heterozygous line). Linkage was detected (LOD=2–13) between the inheritance of the TI pattern and markers associated with linkage group 5 (Ellis et al. 1992). A distance of 6.4 map units (Table 1) was calculated from the linkage between the TI locus (*Tri*) and *Vc-2*, a locus encoding a sub-set of vicilin precursors (Ellis et al. 1986). Analysis of 20 recombinant inbred lines (F_{10}) derived from a cross between JI 126 and JI 430 and of 19 lines (F_{10}) from a cross between JI 15 and JI 61 showed no recombinant or heterozygous types. In the recombinant inbred lines derived from the cross between JI 15 and JI 61, where quantitative differences in 2 TI were scored, linkage to *Vc-2* was again apparent with a map distance of 3.5 map units (Table 1). In neither of the two sets of lines, where alleles at the *r* locus were also segregating, was there any association between the *Tri* and *r* loci (P = 0.2 and 0.8, respectively), although such an association has been inferred by Smirnova et al. (1989).

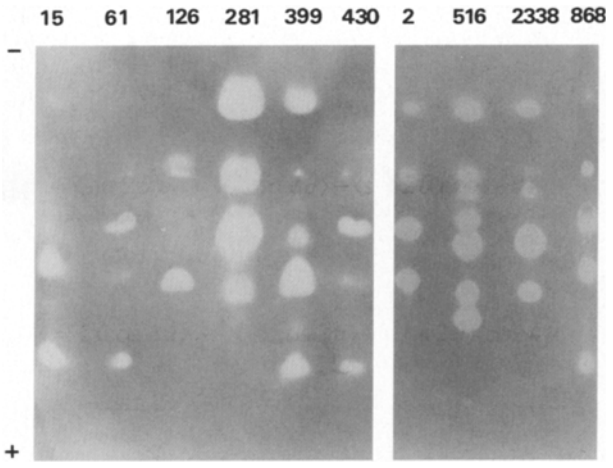

Fig. 1 Non-denaturing polyacrylamide gels of extracts from ten pea genotypes. The *numbers* refer to John Innes germ plasm accessions. Gels were stained for trypsin activity. The *clear zones* represent areas where trypsin is inhibited. The direction of electrophoresis is towards the anode

Mapping quantitative variants for TIA in relation to qualitative TI variants

Although the number of TI apparently varied among lines (Fig. 1), neither the number nor the pattern was correlated with overall TIA. None of the sets of recombinant inbred lines were derived from crosses between genotypes having very different levels of TIA. Crosses were made, therefore, between genotypes at the extremes of the range of variation (Domoney and Welham 1992). The TI pattern of individual small seeds with low TIA could not always be scored reliably because the gel analyses of seed with low TIA requires considerable amounts of meal. Therefore an F_2 population between two large-seeded genotypes facilitated the analysis of individual segregants, and the cross between JI 516 [high TIA; round seeds (*RR*)] and JI 868 [low TIA; wrinkled seeds (*rr*)] and its reciprocal yielded

Table 1 Segregation of TI pattern (A/a) and *Vc-2* or *r* (B/b) among the F_9 lines derived from a cross between JI 281 (A, B) and JI 399 (a, b), the F_{10} lines from crosses between JI 15 (A, B) and JI 61 (a, b) and between JI 126 (A, B) and JI 430 (a, b) and the F_2 population from crosses between JI 516 (A, B) and JI 868 (a, b). The TI pattern was scored from non-denaturing gels as shown in Figs. 1 and 2. *Vc-2* alleles were identified through hybridization analyses, using inserts from pCD4 or pJC2-7; the latter detects several vicilin loci at low-medium hybridization stringency (Ellis et al. 1986). *P*, probability, as determined from a χ^2 analysis of the data shown (TI, *Vc-2*) or data re-grouped into parental and recombinant classes (TI, *r*, F_{10} lines). In the F_2 population, the six classes scored are re-grouped, such that factors are in the coupling phase for the 'dominant' alleles A and B. Recombination fraction (r.f.) and map units (m.u.) were calculated as described by Ellis et al. (1992) for recombinant inbred lines and Stevens (1940) and Kosambi (1944), respectively, for the F_2 population LOD scores (Edwards 1972) determined for TI and *Vc-2* are given

Cross	AB	Ab	aB	ab	*P*	r.f.	m.u.
F_9 (281 × 399) (TI, *Vc-2*)	26	3	2	24	<0.0001 (LOD = 8.58)	0.107	6.392
F_{10} (15 × 61) (TI, *Vc-2*)	10	0	1	5	<0.0005 (LOD = 3.19)	0.063	3.450
F_{10} (15 × 61) (TI, *r*)	7	5	4	3	0.8	–	–
F_{10} (126 × 430) (TI, *r*)	7	2	5	6	0.2	–	–
F_2 (516 × 868) (TI, *r*)	24	16	6	14	0.03	0.334	40.4

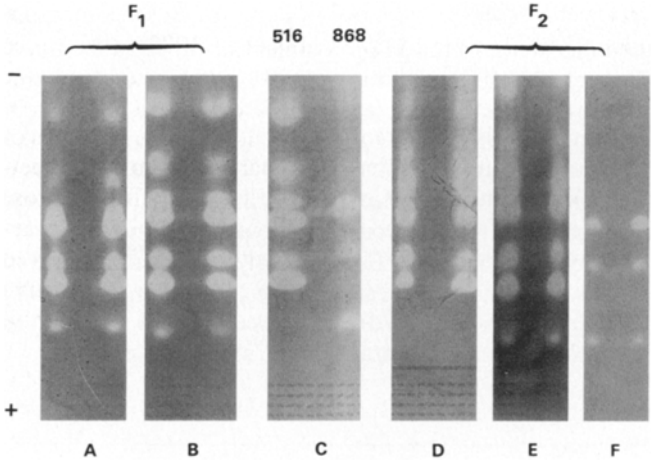

Fig. 2 Non-denaturing polyacrylamide gels of extracts from JI 516 and JI 868 (*panel C*), F_1 seeds derived from a cross between JI 516 and JI 868 and its reciprocal (*panels A and B*, respectively) and F_2 seeds from the same crosses showing the parental (*panels D and F*) and heterozygous patterns (*panel E*). The direction of electrophoresis is towards the anode

the most information. Analysis of F_1 seed indicated an additive TI pattern with the TI of greatest mobility of JI 868 origin being consistently fainter relative to the JI 516 bands (Fig. 2, panel A–C). The TIA of F_1 seed was intermediate between the two parents [6.71±0.82 for F_1 (516 × 868); 8.38±1.34 for F_1 (868 × 516), where the corresponding values for JI 516 and JI 868 were 10.05±0.78 and 2.23±0.27, respectively].

Analysis of the TIA range among 30 F_2 seed from each of the JI 516 × JI 868 cross and its reciprocal showed a very similar distribution for the two sets of data, and hence pooled data are shown in Fig. 3. A spread of TIA between the extremes of the two parental values was evident. Gel analyses of the seed at the extremes of the F_2 TIA distribution showed a clear-cut situation where all of the seed having more than 10 TI units per milligram dry weight showed the JI 516 TI pattern (Fig. 2, panel D). Likewise, all of the seed with TIA in the 0–2 range showed the JI 868 pattern (Fig. 2, panel F). In the intermediate groups of TIA, TI patterns corresponding to those of JI 868 and JI 516 and heterozygous patterns (identified as a JI 516 pattern plus the TI of greatest mobility in JI 868) were detected. However, there was a bias in these distributions, with the JI 516 pattern being identified more frequently among the seed having high TIA and the JI 868 pattern being identified more frequently among the seed having low TIA (Fig. 3 A). It was clear throughout the analyses of the heterozygotes that the TI of greatest mobility in JI 868 was always present as a minor component relative to the TI of JI 516 (Fig. 2, panel E). These data indicate a linkage between the genes encoding qualitative TI variants and the factors affecting overall TIA.

It was evident from these F_2 populations that there was some association between the segregation of the TI pattern or TIA and alleles at the *r* locus (*P*=0.03 for TI pattern and *r*, Table 1). All of the seed in the top TIA group were round, whereas all but 2 in the lowest TIA group were wrinkled (Fig. 3 B). However, no such association was apparent in other F_2 populations (JI 102×JI 868 and reciprocal), nor for TI pattern and *r* in the recombinant inbred lines (see above). A recombination fraction (Stevens 1940) of 0.334±0.077 was calculated from the data obtained for the segregation of the *Tri* and *r* loci in the F_2 population from the crosses between JI 516 and JI 868 (Table 1). These data could be interpreted as a chance event or as being consistent with a presumed close linkage of *Tri* to *Vc-2* (deduced from recombinant inbred lines) and the additive map distances of *Vc-2* to *Lg-1* and *Lg-1* to *r*, which were determined previously in other crosses (Domoney et al. 1986;

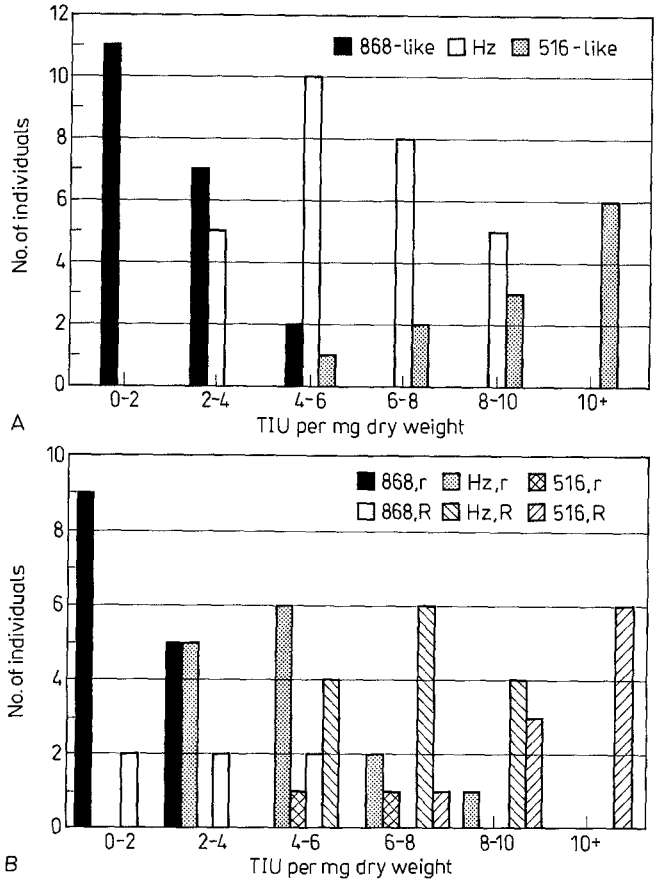

Fig. 3A, B The distribution of TIA [TI units (TIU) per milligram dry weight] among F_2 segregants from the crosses between JI 516 and JI 868 in relation to the segregation of the TI polypeptide pattern (516-like, 868-like or heterozygous (*Hz*) patterns, as indicated) (**A**) or in relation to the segregation of both the TI polypeptide pattern [516, 868 or heterozygous (*Hz*) patterns] and *r* [either RR/Rr (*R*) or rr(*r*)] (**B**)

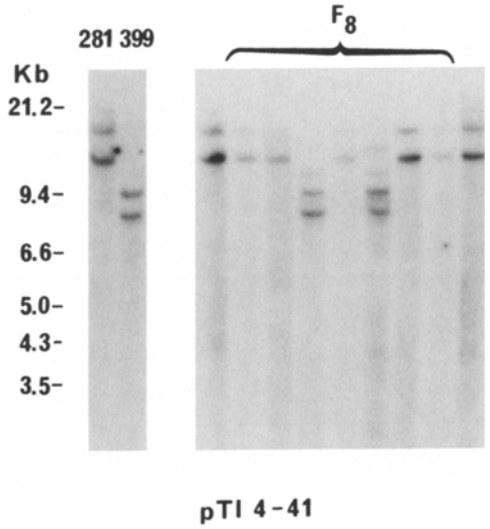

Fig. 4 Hybridization of the insert from the pea TI cDNA clone, pTI4-41, to Southern blots of *Eco*RI-digested DNA from JI 281 and JI 399 and nine recombinant inbred lines (F_8) derived from a cross between JI 281 and JI 399

Ellis et al. 1986). The inconsistent linkage of *Vc-2* to *r*-associated markers in various crosses has been interpreted as being one of the consequences of a possible translocation between linkage groups 5 and 7 (see Ellis et al. 1992). A general effect of the wrinkled-seeded (*rr*) phenotype and its physiological consequences (Wang and Hedley 1991) on TIA is unlikely as we have identified several round-seeded lines with low TIA, and no consistent difference in TIA was detected between pairs of lines that were near-isogenic, except for alleles at the *r* locus (data not shown).

Mapping the genes encoding TI

Hybridization of the TI cDNA insert from pTI4-41 to blots of pea genomic DNA restricted with *Eco*RI showed several polymorphic fragments and, in particular, the fragments which hybridized in JI 281 differed from those in JI 399 (Fig. 4). Analysis of 48 recombinant inbred lines derived from this cross showed only the parental patterns and that the segregation of the DNA polymorphisms correlated with the inheritance of the TI protein variants in 47 of the lines. The remaining line with a JI 281 DNA pattern corresponded to the line for which a heterozygous TI protein pattern had been detected (see earlier), implying that the seed, which was grown for leaf DNA preparation, was a homozygote but was selected from a pool of seed that was segregating at the *Tri* locus. This hypothesis has been verified by analysis of the TI pattern of seed from this line in the next generation. These data are consistent with the TI protein patterns being a direct consequence of variation in the DNA fragments containing the structural genes.

Discussion

We have previously described a ten-fold variation in TIA among pea lines (Domoney and Welham 1992). Here we describe the variation in individual TI polypeptides that exists among a group of pea lines; between 2 and 7 TI were distinguished, but neither the number nor the pattern was related to overall TIA. We have examined the genetics of TI variation to determine the number of loci involved. Very little genetic data exist on TI in other species; in soyabean, for example, it is not clear whether the related but distinct Bowman-Birk, CII and DII inhibitors are the products of one locus (Garcia-Olmeda et al. 1987). Analysis of the CM inhibitors from wheat indicated a co-segregation of expression level with structural variation (see Garcia-Olmeda et al. 1987).

The genetic data presented in this paper suggest that qualitative variation in pea TI polypeptides, quantitative variation in individual polypeptides and overall TIA are all controlled by one or more linked loci on linkage group 5 close to *Vc-2*. The TI structural genes also map to

this position. [For comparison to other pea linkage maps, this linkage group also carries the classical morphological marker *gp* (yellow pods) (see Ellis 1993)]. Processes such as differential glycosylation or other post-translational modification of the TI polypeptides may contribute to TI variation but, if so, the variants which result are likely to reflect amino acid substitutions in the polypeptides. The genetic data have direct implications for breeding programmes and suggest that seed with low or high TIA may be selected from segregating populations through the selection of particular TI or DNA patterns of single seed. In this way, homozygotes can be selected, whereas selections based on assays will give mixed populations. It is clear that analysis of TIA in F_2 populations does not yield the three discrete populations that might be expected from a simple co-segregation of quantitative and qualitative variation. However, the ability to distinguish three such populations would rely on a high degree of accuracy and specificity in the assay being used as well as a lack of environmental effect on the amount of protein synthesized. The assays are subject to some interference by other seed components (such as phenolic compounds and phytate, which may also vary among segregants) and may well be influenced by other segregating genetic factors. Given these provisos, our gel analyses of F_2 segregants are consistent with a simple co-segregation of quantitative and qualitative variants. In the different crosses analysed, a close linkage of *Tri* to *Vc-2* was inferred, although linkage of these to *r* may depend on whether the parents carry a translocation for linkage groups 5 and 7 (see Ellis et al. 1992). If JI 516 has such a translocation, crossover suppression might be expected to occur in the regions affected. For this reason, we cannot categorically state that only one locus is involved in TI variation. However, if there is more than one, linkage can be inferred.

The mapping of TI genes close to *Vc-2* and associated markers has the further implication that, since three patterns of segregation have been described for this linkage group (Ellis et al. 1992), expression of these genes could be greatly influenced by their chromosomal position.

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Note added in proof

The *Tri* locus described here is included in the pea linkage map of Ellis et al. (1993) (Pisum Genetics 25: 5–12), where it is referred to as ti/b. The former designation, *Tri*, is in agreement with the guidelines of the *Pisum* Genetics Association.