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Diversity in inhibitors of trypsin and *Helicoverpa armigera* gut proteinases in chickpea (*Cicer arietinum*) and its wild relatives

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Abstract Developing seeds of eight chickpea (*Cicer arietinum* L.) cultivars (12–60 days after flowering) showed a significant variation in the trypsin inhibitor (TI) and the *Helicoverpa armigera* gut proteinase inhibitor (HGPI) content. For example, the highest TI (198 units/g) and HGPI (23 units/g) activities were exhibited by mature seeds of cv ICCV-2, whereas the lowest inhibitor activities were observed in cv PG8505–7 (96.1 TI units/g) and cv Vijay (5 HGPI units/g). Electrophoretic patterns showed a variation in TI bands during the early stages of seed development, indicating cultivar-specific TI accumulation. Among the seed organs, TI and HGPI activities were highly localized in the embryo-axis as compared to the cotyledons in immature and mature seeds. Moisture stress, as effected under rainfed conditions, resulted in reduced PI levels. Wild relatives of chickpea revealed variability in terms of the number and intensity of TI bands. However, when assessed for inhibition of HGP, none of the wild *Cicer* species showed more than 35% inhibition, suggesting that a large proportion of HGP was insensitive to PIs from *Cicer.* Our results provide a biochemical basis for the adaptation of *H. armigera* to the PIs of *Cicer* species and advocate the need for the transformation of chickpea with a suitable gene(s) for *H. armigera* resistance.

Key words *Cicer* · Developing seeds · Genetic diversity · *Helicoverpa armigera* · Moisture stress · Proteinase inhibitor · Tissue specificity

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Introduction

Chickpea (*Cicer arietinum* L.) is the third most important pulse crop of the world providing high quality protein in a vegetarian diet. The species covers 10.2 million hectares of land and accounts for 7.9 million tons of the world's pulse production (Singh 1997). Apart from human consumption, it is also used as a feed for livestock and contributes substantially to soil nitrogen. The productivity of chickpea is, however, restricted due to its heavy infestation by *Helicoverpa armigera* (Hübner) which feeds on the foliage, flowers, and particularly on developing seeds. A single larva damages several pods per day leading to severe losses in crop yield.

Plants synthesize various proteinaceous and non-proteinaceous compounds against insect attack. Amongst these, proteinase inhibitors (PIs) are the most-studied class of plant-defense proteins. In legumes, PIs accumulate in a large amount during seed maturation, suggesting their role both in the deposition of storage protein and in the plant defense mechanism (Koiwa et al. 1997). Their accumulation in quantities far more than required for inhibiting endogenous proteinases, and in many cases the absence of inhibitory activity against plant proteinases, underlines their role as defense proteins against predators. PIs are induced under various stress-prone conditions such as insect chewing, mechanical wounding, pathogen attack, drought and UV exposure (Schaller and Ryan 1995; Conconi et al. 1996; Giri et al. 1998). PIs are the end products of several defense cascades activated by numerous systemic and non-systemic elicitors, such as systemin, ethylene, methyl jasmonate, absicic acid, salicylic acid, fungal cell wall oligomers, larval oral secretions and electrical and hydraulic signals, leading to increased accumulation in local as well as in remote tissues (Ryan 1990; Wildon et al. 1992; Schaller and Ryan 1995; Korth and Dixon 1997). Co-evolution of plants and insects leads to the formation of isoforms of PIs and of proteinases with different specificities. Plants have a specific advantage for the improved efficacy of their PIs by evolving multi-domain variants (Jongsma and Bolter

1997). Moreover, mutation in PI genes tends to generate an array of allelic variability in the plant genome and, as a result, the plant gene pool is expected to be rich in PI variants showing diverse properties (Ryan 1990). Recently, however, insects have been shown to produce either inhibitor-insensitive proteinases to adapt to their host (Giri et al. 1998) or transgenic plant PIs (Jongsma and Bolter 1997; Michaud 1997)

Chickpea seeds are known to contain PIs and their properties have been studied in detail by Belew and Eaker (1976), Smirnoff et al. (1979), Jibson et al. (1981) and Saini et al. (1992). However, no data are available regarding the biochemical interaction of chickpea PIs with gut proteinases from *H. armigera*, which is a devastating pest of chickpea. Since *H. armigera* is a pest of immature chickpea seeds, the focus of our initial research was to study the expression of chickpea TIs in developing seeds and their induction upon insect chewing (Harsulkar et al. 1997; Giri et al. 1998). It was, however, very important to assess the levels of PIs and their interaction with the gut proteinases of *H. armigera* in several chickpea cultivars that are commonly used in Indian chickpea breeding programs. The pod-damage data of chickpea cultivars (Anon 1995) prompted us to study the expression of PIs in developing seeds of chickpeas showing differences in their susceptibility to *H. armigera* infestation. We selected eight elite cultivars which are extensively used for chickpea breeding in India. Among these cultivars, five (Vijay, Vishal, ICCV2, ICCV10 and PG8505–7) are less susceptible and three (Vishwas, PG91028 and PG8404–1) are highly susceptible to *H. armigera* attack (Anon 1995). It is well known that the wild germplasm contains useful genes that may not be present in cultivars (Singh and Ocampo 1997). Therefore, wild relatives of chickpea were analyzed for PIs and their potential to inhibit *H. armigera* gut proteinases (HGPs). Since chickpea is often grown under water-limiting conditions in the Indian sub-continent (Saxena 1987), and the expression of PIs has been shown to alter by drought (Brzin and Kidric 1995), it was thought important to study PI expression in developing chickpea seeds exposed to moisture stress. Two cultivars, Vishal and Vijay, were grown under conditions creating terminal drought and the accumulation of PIs in developing seeds was examined. In addition, the distribution of PI activity in the embryo-axis and cotyledons during the stages of seed development was studied.

Materials and methods

Plant material

Eight cultivars of chickpea (Vijay, Vishwas, Vishal, PG91028, ICCV2, ICCV10, PG8505–7 and PG8404–1) were grown in a randomised block design in quadruplicates under irrigated conditions at the Pulses Research Station, Mahatma Phule Krishi Vidyapeeth (MPKV), Rahuri. Chickpea flowers were tagged on the day they opened and developing pods were harvested 12, 24, 36, 48 and 60 days after flowering (DAF). Two chickpea cultivars, Vijay and Vishal, were grown under rainfed conditions (without irrigation) and developing pods were collected 5, 10, 15, 20, 25, and 35 DAF. Seeds of wild *Cicer* species were obtained from the International Crop Research Institute for Semi-Arid Tropics (ICRISAT), Patancheru, India, and from Dr. Fred Muehlbauer of Washington State University (WSU), Pullman, USA. The frozen tissues were ground in a pestle and mortar and the dry seeds in a mixer-blender. The homogenate was dehydrated and de-pigmented by washing at least six times with acetone followed by hexane. The solvents were removed by filtration and the tissue powders were air-dried.

Extraction of PIs and HGP

The seed powders were mixed with 10 vol of distilled water and kept overnight at 4°C for extraction with intermittent shaking. The suspension was centrifuged at 10,000g for 20 min at 4°C and the supernatant was stored in aliquots at −20°C. Fourth-instar larvae of *H. armigera* were dissected and isolated mid-guts were stored frozen at −70°C. As and when required, the gut tissue was homogenized in 3 vol of 0.2 M glycine-NaOH buffer pH 10.0 and kept for 2 h at 10°C. The suspension was centrifuged at 10,000 g for 20 min and the supernatant was used as a source of HGP. The protein concentration of the extracts was quantified as described by Bradford (1976).

Estimation of PIs

TI and HGPI activities were measured using the method described earlier (Giri et al. 1998). Appropriate volumes of the chickpea seed extract, enough to give 40–60% inhibition of trypsin (20–35% inhibition in the case of HGP), were mixed with 15 µg of trypsin or an equivalent amount of HGP and allowed to stand for 15 min at 27°C. The residual proteinase activity was measured by incubating the seed extract with synthetic substrate benzoyl arginine-*p*-nitroanilide (BA*p*NA) for 10 min at 37°C. One unit of proteinase activity was defined as the amount of enzyme that caused an increase of 1 optical density unit at 410 nm due to the release of *p*-nitroaniline. One PI unit was defined as the amount of inhibitor that inhibited 1 unit of proteinase activity.

Electrophoretic visualization of TIs

TI isoforms were detected by using either the gel-X-ray film contact-print technique (Pichare and Kachole 1994) or by gelatin-polyacrylamide gels (Felicioli et al. 1997). After electrophoresis, the gel was incubated in 0.1 M Tris-HCl buffer (pH 7.8) for 10 min followed by incubation in 0.1% trypsin for 15 min. The gel was washed and placed on a piece of X-ray film. After 2–5 min, the gel was removed and the X-ray film was washed gently to remove the hydrolyzed gelatin. TI activity bands were visible as unhydrolyzed gelatin. The film was then developed and contact printed. The gelatin-polyacrylamide gel was incubated in trypsin solution for 1 h and stained with Commassie Brillant Blue R-250 (Felicioli et al. 1997).

Results

Accumulation of PI activity during seed development in different chickpea cultivars

The protein content, TI and HGPI activity of eight different cultivars of chickpea were determined at five developing stages, namely 12, 24, 36, 48 and 60 DAF (Table 1). Protein accumulation took place at a rapid pace after the initial lag phase and increased with seed maturity. The period of maximum protein accumulation for all the

Table 1 Protein and PI content in the developing seeds of eight different chickpea cultivars. The values are the average of four replicates

Cultivars	12 DAF	24 DAF	36 DAF	48 DAF	60 DAF
Vijay					
Protein	0.9	2.3	14.5	37.5	40.0
TI units/g	ND[a]	5.4	38.9	76.5	136.7
HGPI units/g	ND	5.4	5.5	5.9	5.0
Vishwas					
Protein	6.3	8.2	16.2	19.42	28. 6
TI units/g	ND	25.7	74.8	118.2	104.3
HGPI units/g	ND	3.9	6.4	7.4	7.6
Vishal					
Protein	2.2	1.7	6.6	14.4	27.1
TI units/g	ND	17.2	37.9	81.6	130.5
HGPI units/g	ND	1.3	4.4	5.3	8.7
PG91028					
Protein	3.3	3.7	7.5	25.9	33.4
TI units/g	ND	13.4	48.4	101.7	127.2
HGPI units/g	ND	2.9	2.8	12. 7	11.2
ICCV2					
Protein	2.5	3.0	32.0	41.2	39.6
TI units/g	ND	43.5	67.7	60.2	198.0
HGPI units/g	ND	7.2	8.5	13.7	23.1
ICCV10					
Protein	5.5	3.8	16.0	18.6	27.6
TI units/g	ND	10.4	54.9	147.7	165.1
HGPI units/g	ND	3.5	5.3	5.4	6.7
PG8505–7					
Protein	0.9	2.7	19.8	28.2	26.2
TI units/g	ND	15.5	69.2	101.8	96.1
HGPI units/g	ND	2.3	12.8	12.2	11.6
PG8404–1					
Protein	1.2	1.6	4.2	17.1	28.8
TI units/g	ND	7.9	73.4	120.2	163.4
HGPI units/g	ND	0.86	2.4	6.8	8.9

[a] ND, not detectable

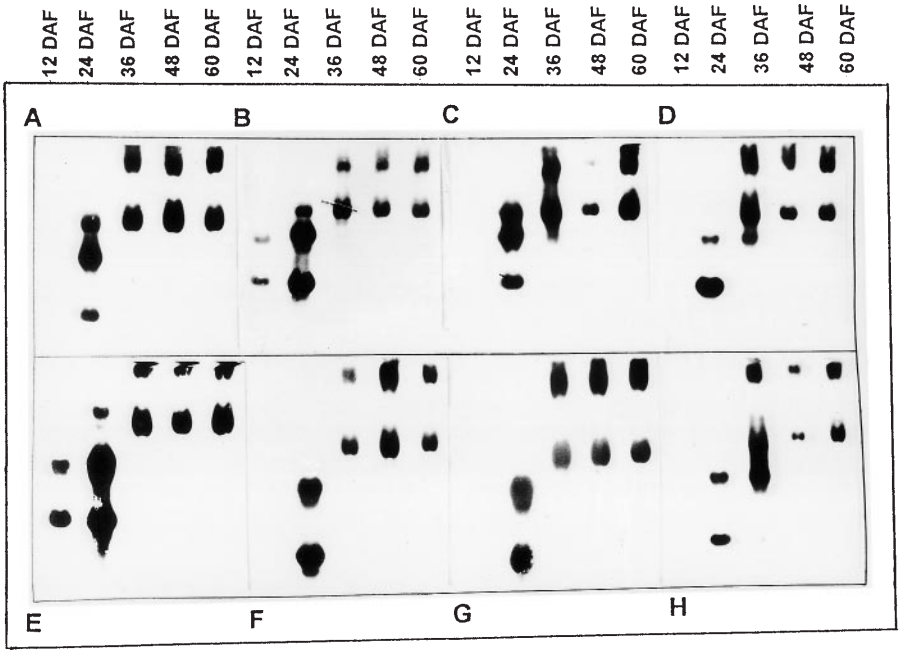

Fig. 1 TIs in eight chickpea cultivars through five stages of seed development. Electrophoresis was carried out on 10% non-denaturing polyacrylamide gels with a discontinous buffer system. TI activity bands were visualized by the gel-X-ray film contact-print technique (details as described in Materials and methods). An equal quantity of protein (30 µg) was loaded in each lane. For 12 DAF extracts, the maximum amount of protein (60 µg) was loaded as no activity was detected in spectrophotometric assays. Lanes 1–5, 12, 24, 36, 48 and 60 DAF, respectively. *A*-ICCV10, *B*-Vijay, *C*-PG8404–1, *D*-Vishal, *E*-ICCV2, F-Vishwas, *G*-PG8505–7 and *H*-PG91028

cultivars was between 24 and 48 DAF. TI activity was not detectable at the 12 DAF stage in any of the cultivars. It attained detectable levels at 24 DAF with a progressive increase untill seed maturation. The cvs Vijay, Vishwas, PG91028, ICCV10, PG8505–7, and PG8404–1 showed a higher rate of TI accumulation from 24 to 36 DAF. Cv Vishal showed steady rates of TI increase from 24 DAF to 60 DAF. Cv ICCV2 was unique in exhibiting the highest amount of TI and HGPI activity at the 24 and 60 DAF stages. As compared to TI activity,

Table 2 Distribution of PI activity and storage proteins in different organs of developing and mature seeds of chickpea cv Vijay. The values are the average of three replicates

Seed organ	Protein (mg/g)		TI units/g		HPGI units/g	
	Mid-mature	Mature	Mid-mature	Mature	Mid-mature	Mature
Pod-cover	0.24	0.187	ND[a]	ND	ND	ND
Seed-coat	0.21	0.375	ND	ND	ND	ND
Cotyledon	11.25	31.87	21.54	237.5	10.6	57.8
Embryo-axis	52.15	42.85	181.62	336.68	87.0	104.6

[a] ND, not detectable

HGPI activity was very low but exhibited a wider range of variation in different cultivars through the various stages of seed development. At 12 DAF, no HGPI activity was detected in any of the cultivars, while at 24 DAF the cultivars showed HGPI activity ranging from 0.86 to 7.2 units/g. Cv Vijay was found to have a higher HGPI activity at 24 DAF (5.4 units/g) and remained constant untill 60 DAF (5 units/g), which was the lowest as compared to the other cultivars. The cv PG 91028, ICCV2 and PG8505–7 showed a higher amount of HGPI activity at 60 DAF as compared to the other cultivars.

Electrophoretic profiles of TI isoforms in developing chickpea seeds

Figure 1 depicts the electrophoretic patterns of TIs in chickpea cultivars through five stages of seed development. All chickpea cultivars showed the presence of two fast-moving TI isoforms specific to early stages of seed development (12–36 DAF) and two slow-moving isoforms characteristic of the mature seed stages. Between these two stages there was a transition stage where the fast- and the slow-moving forms co-existed. The cvs ICCV10, Vijay, PG8404–1 and ICCV2 revealed the maximum number of TI bands at 24 DAF (Fig. 1A, B, C and E). However, the transition stage in which early and late stage-specific TIs showed an overlap was either absent or was too transient to detect in Vishwas and PG8505–7 (Fig. 1F and G). Cvs Vijay and ICCV2 (Fig. 1B and E) could be distinguished from other cultivars as they exhibited TI activity bands at 12 DAF stage, although no TI activity was detected in solution assays. This can be attributed to the higher sensitivity of the gel-X-ray film contact-print technique over spectrophotometric assay. Ambekar et al. (1996) have reported the presence of TI activity bands in pigeonpea during seed development using the same technique, although there was no detectable TI activity in the caseinolytic assay. None of the other chickpea cultivars showed any detectable TI activity at 12 DAF by either of the assay methods.

Distribution of TI activity in seed organs

The distribution of TI activity and storage proteins was studied in different seed organs of mid-mature and mature seeds of cv Vijay (Table 2). The embryo-axis exhibited a considerably higher protein content than the cotyledon at mid-maturation. More importantly, the embryo showed about nine-fold more TI and HGPI activity than the cotyledon at this stage. However, the rate of increase in PI activity in the cotyledon was considerably more than that in the embryo-axis as the seed matured (Table 2). The embryo-axis showed a higher specific activity for PI than the cotyledon indicating a higher PI deposition in the embryo-axis. At mid-maturation, 52% TI activity was localized in the cotyledon and 48% in the embryo-axis (Fig. 2). However, in the mature seed the cotyledon contributed to 91% of the TI activity as against only 9% by the embryo. When the seed organs were measured for their contribution to fresh weight at the mid-mature stage, the share of the cotyledon was 68%, the seed coat 31.3% and the embryo-axis 0.7%. In the mature seed it shifted to 84% cotyledon, 14.7% seed coat and 1.2% embryo-axis (Fig. 2).

Changes in PI activity under the influence of moisture stress

When the two chickpea cultivars Vijay and Vishal were grown under rain-fed conditions, the specific changes included the following: (1) shortening of the seed maturation period from 60 DAF to 35 DAF, (2) a greater accumulation of protein between 25 to 35 DAF (3) a higher protein content in the mature seeds, and (4) a decrease in TI activity as compared to irrigated plants (Table 3).

TI variants in Cicer species

Figure 3 shows the extent of variability in TI isoforms in seeds of wild *Cicer* species. The wild species exhibited diversity in TI isoforms with respect to both number and activity as compared to chickpea. Accessions of the same *Cicer* species obtained from WSU (USA) and ICRISAT (India) exhibited differences in TI profiles. For example, *Cicer pinnatifidum* (WSU) showed inhibitor activity bands (Fig. 3, lanes 4) while the same species obtained from ICRISAT did not possess any TI band (lane 17). Similarly, *Cicer echinospermum* obtained from WSU and ICRISAT showed variation in TI profiles (lanes 2 and 15). Three accessions of *Cicer bijugum* obtained from ICRISAT and one accession from WSU also revealed variation in TI patterns. In the case of *Cicer bijugum* (WSU) two major bands and one minor activity band were detected (lane 3). Accession ICCW 42# 200 exhibited at least three TI bands of high intensity (lane 10) while accessions ICCW 72 LWC 42–2 and ICCW 42# 201 showed one major and a minor TI band (lanes 11 and 12).

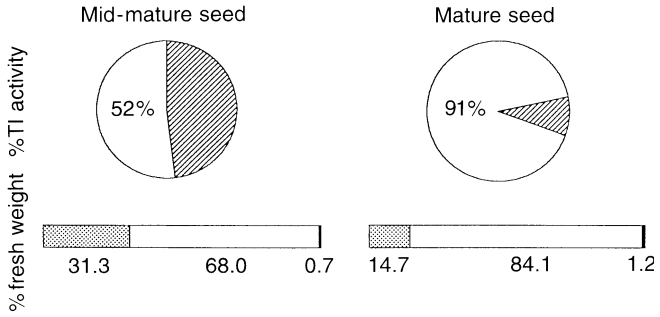

Fig. 2 Distribution of TI activity in seed organs, cotyledon (□) and embryo axis (▨), of mid-mature and mature seed of chickpea cv Vijay. The percent values of TIs were calculated as per the fresh weight contributed by the respective seed organs. The *bar graphs* show the percent distribution of the fresh weight of seed organs. The seed coat (▤), cotyledon (□) and embryo (■) of mid-mature and mature seeds were separated and the percent fresh weight contributed by each of them was calculated

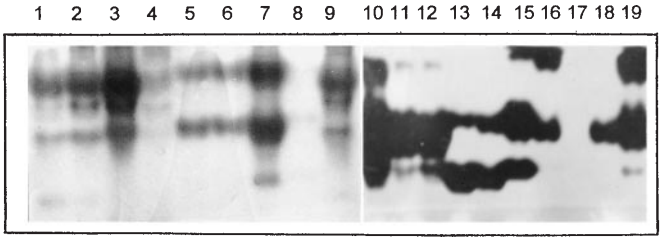

Fig. 3 TI profiles of wild relatives of chickpea. In each lane 30 µg of seed protein was loaded, while in cases where less, or no, TI activity was detected 50 µg of protein was loaded. *Lanes 1–9* (*Cicer* wilds obtained from WSU) and *lanes 10–19* (wilds from ICRISAT). *Lanes 1 and 16 C. reticulatum*; *lanes 2 and 15 C. echinospermum; lane 3 C. bijugum*; *lane 10 C. bijugum* (ICCW 42# 200), *lane 11 C. bijugum* (ICCW 72 LWC 42–2); *lane 12 C. bijugum* (ICCW 42#201)*; lanes 13 and 14 C. judaicum; lanes 4 and 17 C. pinnatifidum; lane 5 C. oxyodon; lane 6 C. microphyllum; lane 7 C. anatolicum; lane 8 C. songaricum; lanes 9 and 19 C. arietinum; lane 18 C. cuneatum*

Table 3 Effect of moisture-stress on protein and PI content in developing seeds of chickpea cv Vishal grown under rainfed conditions. The values are the average of three replicates

Stage (DAF)	Protein (mg/g)		TI units/g		HGPI units/g	
	Vijay	Vishal	Vijay	Vishal	Vijay	Vishal
5	5.7	4.62	ND[a]	ND	ND	ND
10	8.3	6.25	ND	ND	ND	ND
15	9.4	6.37	ND	ND	ND	ND
20	15.32	16.97	11.05	17.73	1.3	1.58
25	17.58	18.55	17.58	22.86	2.7	2.84
30	37.80	39.37	32.96	65.37	3.7	6.60
35	58.70	39.87	54.10	77.21	3.2	5.11

[a] ND, not detectable

Table 4 Inhibition of *H. armigera* gut proteinase activity by PIs from mature seeds of chickpea (*C. arietinum*) and its wild relatives. The amount of inhibitor which showed 100% inhibition of trypsin was chosen for assessing the inhibition of HGP. The values are the average of four replicates

Genotype	Source	Inhibition of HGP (%)
C. arietinum (cv Vijay)	ICRISAT	33
C. bijugum (ICCW41 #200)	ICRISAT	29
C. bijugum (ICCW72 LWC42–2)	ICRISAT	36
C. bijugum (ICCW42 #201)	ICRISAT	28
C. judaicum (ICCW 33)	ICRISAT	23
C. judaicum (ICCW92 LR126)	ICRISAT	24
C. echinospermum (ICCW44 #204)	ICRISAT	33
C. reticulatum	ICRISAT	28
C. pinnatifidum	ICRISAT	7
C. cuneatum	ICRISAT	2
C. pinnatifidum	WSU	23
C. oxyodon	WSU	21
C. anatolicum	WSU	25
C. songaricum	WSU	12
C. microphyllum	WSU	18
C. echinospermum	WSU	5
C. reticulatum	WSU	21
C. bijugum	WSU	30

Cicer oxyodon (lane 5) and *Cicer microphyllum* (lane 6) gave very similar TI band patterns. *Cicer reticulatum* (lanes 1 and 16) was characterised by two bands, as in chickpea (lane 9). *Cicer anatolicum* and *Cicer echinospermum* showed three TI bands of the same mobility, whereas no TI band was detected in *Cicer songaricum*. Both accessions of *Cicer judaicum* revealed two TI activity bands differing in their mobility (lanes 13 and 14). *Cicer cuneatum* showed a single TI activity band (Lane 18).

Insensitivity of HGP activity to *Cicer* PIs

The amount of seed extract required to inhibit 100% trypsin activity was titrated against HGP. The results are summarized in Table 4. The highest inhibition of HGP (36%) was effected by *Cicer bijugum* PIs (Accession ICCW 72 LWC 42–2), followed by 33% in *Cicer echinospermum* and *Cicer arietinum* (cv Vijay). The HGP inhibition by other *Cicer* species including chickpea ranged between 2

and 30%. The lowest HGP inhibition was detected in seed extracts of *Cicer pinnatifidum* (ICRISAT) (7%) and *Cicer cuneatum* (2%). *Cicer pinnatifidum* obtained from WSU showed a 23% inhibition of HGP.

Discussion

Variation in PIs of chickpea cultivars during seed development and in *Cicer* species

The differential expression of TI forms can be attributed either to temporal expression of gene groups or to post-translational modifications of PIs (Domoney et al. 1994, 1995; Harsulkar et al. 1997; Giri et al. 1998). Domoney et al. (1995) reported the generation of multiple TI forms in the pea from two primary gene products. Giri et al. (1998) have shown that chickpea TI-5 is the proteolysis product of chickpea TI-1. The variation observed at the transitory stage among the chickpea cultivars points to considerable variability in the expression and/or modification of TI proteins. No strong correlation has been found between the pod-damage data (Anon 1995) and the PI content at different stages of seed development. This can be attributed largely to the insensitivity of HGP towards chickpea PIs and the strong feeding preference of *H. armigera* in the field. The latter is evident when *H. armigera* has a choice of host, and this might explain the inconsistency revealed in the pod-damage data. More interestingly, HGPI activities vary widely among cultivars, with ICCV2 showing highest HGPI activity in the mature seeds (Table 1). Obviously, however, this level of HGPI activity is not sufficient to afford protection to chickpea against *H. armigera*.

The TI isoforms of wild *Cicer* species have revealed significant variation while there is a greater conservation of TI isoforms in the mature seeds of the chickpea cultivars (Fig. 3). A similar observation exists in pigeonpea where TIs and chymotrypsin inhibitors are conserved in mature seeds of the cultivated pigeonpea whereas a high level of diversity exists in the uncultivated species of *Cajanus* (Kollipara et al. 1994; Pichare and Kachole 1996). The variation observed in the wild *Cicer* species is considered significant, as the TIs are known to serve as defense proteins against herbivores (Ryan 1990). *Cicer reticulatum* and *Cicer arietinum* show similar TI band patterns, which suggests that *Cicer reticulatum* is genetically closer to *Cicer arietinum*. This is in good agreement with the karyotype and crossability data, as well as the seed storage protein-analysis and isozyme variation reported earlier, and corroborates the conclusion that *Cicer reticulatum* is the presumed progenitor of *Cicer reticulatum* (Ladizinsky and Adler 1976; Kazan and Muehlbauer 1991; Tayyar and Waines 1995).

When the effectiveness of the wild *Cicer* PIs was assessed against HGP by in vitro assays, none of them showed more than 36% inhibition (Table 4). *H. armigera* is a polyphagous pest and possesses a population of proteinases in its gut (Bown et al. 1997; Harsulkar et al. 1998). HGP activity is not only insensitive but also possesses the ability to digest chickpea TIs (Giri et al. 1998). The ineffectiveness of *Cicer* wild PIs indicates that *H. armigera* is adapted to a wide range of PIs. Field screening for resistance to *H. armigera* in chickpea and its wild relatives did not identify any good candidates offering substantial resistance against the insect pest.

Tissue specificity of PIs

Various reports have shown that the seed expresses a unique set of seed-specific proteins (Gatehouse et al. 1986; Goldberg et al. 1989), which are expressed almost exclusively during embryogenesis and are temporally and spatially regulated in the seed organs. The seed-specific proteins include embryo-axis-specific proteins (expressed throughout embryogenesis) and seed-storage proteins, lectins and PIs that are expressed from mid-maturation to late-maturation stages of embryogenesis (Goldberg et al. 1989). PIs accumulate during seed/tuber maturation suggesting that they facilitate the accumulation of seed-storage proteins (Koiwa et al. 1997). The present study shows that the embryo-axis possesses a very high level of PI activity even in immature seeds as compared to the cotyledons. Recently, Welham et al. (1998) have demonstrated the immunolocalization of TI activity in the embryo-axis in developing, as well as germinating, seeds in *Pisum.* The high localization of PI activity in the embryo-axis may be attributed to their defensive properties, which are utilized for protection of the embryo from insect-pests. The PI activity of the seed increases with maturity; however, the increase is greater in the cotyledons than in other tissues. The cotyledons, which contribute to 68% of the seed weight at mid-maturation, increase in weight to 84% at maturity. Since the embryo shows very high specific activities of both TIs and HGPIs (Table 2), isolation of promoters which express genes of anti-feedent proteins for developing resistance to *H. armigera* in chickpea would be of considerable interest.

Moisture-stress vis-a-vis the synthesis of PIs

Chickpea is grown in dry areas on conserved soil moisture and in areas where extremes of temperature persist during the lifecycle of the plant (Singh 1997). When chickpea cultivars are grown under rainfed (as against irrigated) conditions, the seed maturation period decreases from 60 DAF to 35 DAF (our field observations). Kollipara et al. (1994) have found that the seed-maturation period decreases by growing long-duration pigeonpea cultivars at locations different from their area of adaptation. Early maturity allows the plant to escape from drought and is found to be beneficial for chickpea cultivation in peninsular India. The response to supplemental irrigation by chickpea is substantial and often doubles the yield under optimum soil conditions (Saxena 1987). The present work has revealed an increase in the seed protein content and a lowered PI activity in both of the

cultivars which are subjected to moisture-stress. This is, however, accompanied by a lowered yield of the crop (data not shown). The increase in protein content in both cultivars of chickpea under rain-fed conditions may be a response to the stress stimulus. Higher protein accumulation in legume plants exposed to drought at the seed-filling stage may be at the cost of carbohydrate deposits. In legume plants exposed to terminal drought conditions, there is a rapid mobilization of carbohydrates from the leaves and stem towards the seed (Subbarao et al. 1995), which is possibly diverted to a greater protein synthesis. However, further investigations are needed to arrive at any definite conclusion. Moreover, it has been demonstrated that under conditions of stress, drought-sensitive cultivars of *Phaseolus* and *Vigna* show increases in proteolytic activity possibly depleting protein deposits, whereas the drought-resistant cultivars, on the other hand, show a decrease in proteolytic activity and an increase in PI content (Brzin and Kidric 1995). Increase in PI activity in the seeds of the pigeonpea cultivars grown in different locations from their area of adaptation was also reported by Kollipara et al. (1994).

In summary, our work indicates that considerable variability exists in the chickpea PIs (1) during seed maturation in different cultivars, (2) in response to moisture stress, (3) with respect to localization in different seed organs, and (4) in wild *Cicer* species. It is also evident that although there is significant variability in the inhibitory activity against HGP in chickpea and in its wild relatives, none of them can inhibit HGP activity totally to offer protection against *H. armigera*. The potential of PIs of chickpea and its primary gene pool to develop resistance against *H. armigera* is limited, thus emphasizing the need for the genetic transformation of chickpea with suitable heterologous PI(s) to counteract the mid-gut proteinases of *H. armigera*.

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