HETEROGENEITY AND NATURAL VARIABILITY OF PROTEINASE INHIBITORS IN SUNFLOWER (*Helianthus* L.) AND OTHER COMPOSITAE

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Summary: Multiple molecular forms of proteinaceous inhibitors of digestive proteinases of animals and extracellular proteinases of phytopathogenic fungi were identified in seeds of wild and cultivated *Helianthus* and other *Compositae* species using simple and effective techniques. *H. annuus* seeds contain at least two types of trypsin inhibitors (TI) and bifunctional trypsin/subtilisin inhibitors (T/SI). The main TI, characteristic of the majority of *Helianthus* and some *Tithonia* species, is unique among plant proteinase inhibitors in its small size (1,500) and cyclic structure while a second TI also has a low mass (about 2,500). The TIs and T/SIs vary widely in *H. annuus* lines and wild *Helianthus* species in their presence or absence, composition and pI. Analysis of F_2 hybrids indicated that the three loci encoding T/SI components were linked. Similar components were found in annual diploid species with the B genome but not in perennials with the A genome. The T/SI present in seeds and vegetative organs were active against extracellular proteinases of the white rot fungus *Sclerotinia sclerotiorum*, an important pathogen of sunflower, indicating a possible protective role. T/SI type inhibitors are also widely distributed in other Compositae species, being present in representatives of the subfamilies *Carduoideae* (genera *Carthamus, Centaurea, Cirsium*) and *Cichorioideae* (*Lactuca, Taraxacum*) and some tribes of the subfamily *Asteroideae* (*Heliantheae*, genera *Gaillardia*, *Helianthus*, *Rudbeckia*) etc. Some representatives of the tribe *Heliantheae* (*Cosmos*, *Zinnia* and *Gaillardia*) contained very highly active and heterogeneous TIs and SIs. Studies of the polymorphism, distribution and natural variability of proteinase inhibitors can provide information on the evolution of protective protein systems and the mechanisms of resistance to pathogenic organisms in sunflower and other species of the Compositae.

1. Introduction

The seeds and vegetative parts of higher plants contain various proteinaceous inhibitors of insect, fungal, mammalian and endogenous proteinases. The inhibitors may be involved in plant defence systems against harmful organisms and may also play regulatory roles during plant development (Shewry & Lucas, 1997; Kumar *et* al. 1999). Furthermore, plant inhibitors are of interest in relation to problems of host/parasite co-evolution (Konarev, 1996), as markers in studies of plant diversity and evolution (Konarev 1982, 1999b; Kollipara & Hymowitz, 1992) and as potential drugs with antiviral and other properties. Genes encoding potent and stable inhibitors can be transferred to other plants to improve their pest or fungal resistance (Ryan, 1990). Proteinase inhibitors are well studied particularly in the families *Fabaceae, Poaceae* and *Solanaceae*. Some 12 families of inhibitor can be recognised based on their amino acid sequences and target proteinases (Shewry, 1999). Until recently, inhibitors in sunflower and other *Compositae* remained unstudied.

We have, therefore, (i) determined the polymorphism, variability, genetic control and biochemical properties of proteinase inhibitors in lines and varieties of cultivated sunflower *Helianthus annuus* L. and other cultivated and wild *Helianthus* L. species; (ii) studied the distribution and heterogeneity of the main inhibitor types in various *Compositae* groups, (iii) compared the sunflower inhibitors with those of other *Compositae* and (iv) searched for novel inhibitors. Special attention has been given to inhibitors of trypsin, which is a typical digestive enzyme of insects, mammals and fungi, and subtilisin, a proteinase of microorganisms.

2. Materials and methods

Seeds and leaves. Seeds and fresh or dry leaves of sunflower *Helianthus* L. and various *Compositae* species were obtained from world collection of the Vavilov Institute of Plant Industry (VIR, St. Petersburg) and the herbarium of the Komarov Botanical Institute (St. Petersburg) or were collected by authors. The material included 14 varieties of different origin and 70 inbred lines of *Helianthus annuus* L. selected on the basis of various morphological and other features for 7-18 generations. Artificial pollination was carried out to produce F_1 and F_2 hybrid seeds. Wild *H. annuus* included ssp. *annuus* and 3 accessions of ssp. *lenticularis*. Other diploid, tetraploid and hexaploid species are listed in text or figures.

Other Compositae were represented by 110 species from subfamilies *Carduoideae* (8 genera), *Cichorioideae* (9 genera), and *Asteroideae* (37 genera). Main taxa were specified according to K.Bremer (1996). Herbarium seed and leaf material after 1960 was used.

Proteins. Proteins were extracted with water (1:3 w/v) from seeds or leaves after milling and then were separated by isoelectric focusing (IEF) in Servalyt precotes gels (Serva) (Konarev *et al*., 2000) or by thin layer gel-filtration (TLGF) (Konarev, 1982). Proteinase inhibitors were detected by the gelatin replicas method (Konarev, 1986; Konarev *et al*., 2000). Single inhibitor components were purified by affinity chromatography and reversed-phase HPLC (Konarev *et al*., 2000).

3. Polymorphism of serine proteinase inhibitors in sunflower

IEF of proteins extracted from seeds of several varieties (var.) and lines of sunflower (*H. annuus*) followed by detection of inhibitors with the gelatin replicas method revealed seven main band positions (Fig. 1, a-f & h) when replicas were developed by trypsin (C, 6-8) and five ones when subtilisin was used (B). Bands “a-f & g” were active against both proteinases while “a” and “b” inhibited trypsin only (band “b” was a doublet). Since inhibitor components corresponding to band “a” were much more active than others they were analyzed separately. Fig. 1 A illustrates the variability of band “a” components in various accessions of *H. annuus*, including lines and wild forms. It was present in *H. annuus*, other diploid annual species including *H. petiolaris* Nutt., *H. debilis* Nutt., *H. nuttallii* Torr. ex A. Gray and *H. praecox* Engelm. et Gray (not shown), in all studied tetraploid and hexaploid *Helianthus* species and in

2 accessions of *Tithonia diversifolia* (Hemsl.) A.Gray. Some *H. annuus* lines (VIR-369 and VIR-848b) and single seeds of one of two *H. hirsutus* Rafin. accessions lacked band "a". Analysis of F_2 hybrids between various lines showed monogenic control of this inhibitor (Konarev *et al*., 1999b, 2000). This main sunflower seed trypsin inhibitor (SFTI) was absent from *H. annuus* leaves and heads and from *H. tuberosus* L. tubers. When purified, SFTI appeared to be unique among plant proteinase inhibitors. It has an extremely low M_r (1500) (Konarev *et al*., 1999b, 2000) and cyclic structure (Luckett, *et al*., 1999). TLGF in nondenaturating conditions showed identity of native and purified SFTI by M_r (Fig. 3, 16) indicating the absence of significant modifications of the protein during purification. Because it is potent and stable to proteolysis SFTI is interesting as a model for studying the mechanisms of inhibitor action and as a potential target for transgenic expression in plants or for medical applications. The double band "b" corresponds to TI with a higher M_r (2500) and lower hydrophobicity than SFTI.

Bifunctional trypsin and subtilisin inhibitors (T/SI) are highly variable in sunflower lines, varieties and species (Fig. 1 B, bands "c-h"). Seeds of var. Gigant vary in the presence/absence of the main band "e"(1-5). Lines (6-9) vary in bands "c-f" and "g". Analysis of F_2 hybrids between several pairs of lines showed that bands "d, e & f" are controlled by three linked loci, locus for "e" being located between loci for "d" and "f" (Konarev *et al*., 1999b, 2000).

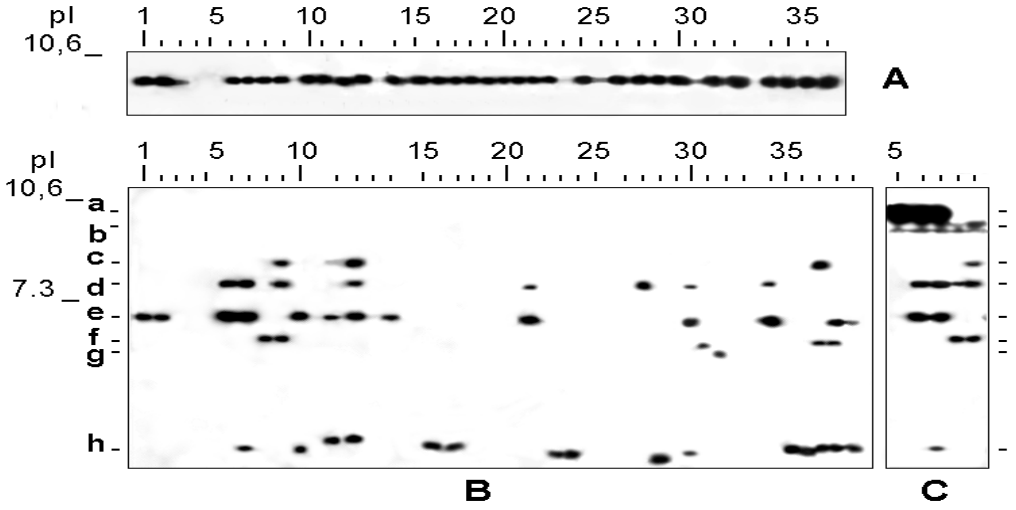

Fig. 1. Polymorphism of serine proteinase inhibitors in cultivated and wild ***Helianthus*** **L**. species. Seed proteins extracted with water were loaded on the gel (in volumes of 0.3 μl for **A** and 5 μl for **B** and **C**) and separated by isoelectric focusing (IEF) in the pH range 3-10 with a distance between electrodes of 11 cm. Trypsin (**A** & **C**) and subtilisin (**B**) inhibitors were detected by the gelatin replicas method. a – h, main positions of inhibitor bands. 7.3 and 10.6, positions of pI markers, horse myoglobin and cytochrom c.
A. Slots **1**, **2**, **4** – **9**, **15**, **30** *H. annuus*: **1 & 30**, line VIR-104; **2**, var. Sunbread 246; **4**, VIR-648b; **5**, VIR-369. **6** – **9** , wild forms: **6**, ssp. *annuus*; **7** – **9** ssp. *lenticularis.* **15**, VIR-130. **3** and **31**, *Tithonia diversifolia.* **10** – **14** & **16** – **21** - diploid perennial species: **10**, *H. giganteus*; **11**, *H. salicifolius*; **12 & 13**, *H. occidentalis* ssp.*occidentalis* and ssp. *plantagineus*, **14**, *H. microcephalus*; **16** & **17** *H. mollis*; **18**, *H. maximilianii*; **19**, *H. grosseserratus*; **20** & **21** *H. divaricatus*. **22** – **29**, **32** & **33**, tetraploid species: **22** – **27**, *H. hirsutus* (**22** & **27** mixtures of seeds; **23** – **26** single seeds of the same accessions as in slot **22**); **28**, *H. decapetalus*; **29**, *H. strumosus*; **32**, *H. laetiflorus*. **33** – **37**, hexaploid species: **33**, *H. californicus*; **34**, *H. rigidus*; **35**, *H. resinosus*; **36**, *H. multiflorus*; **37** – *H. tuberosus*.
B & **C**. Slots **1** – **14**; **21**, **30, 31 & 34** *H. annuus*: **1-5**, var. Gigant, single seeds; **6** , **21 & 34**, VIR-130; **7 & 30** VIR-104; **8 &31**, VIR-369; 9, VIR-648b; **10**, var. Sunbread 246; **11-14**, wild forms: **11**, ssp. *annuus*; **12 - 14,** ssp. *lenticularis*. **15 –20 & 22 – 25**, diploid perennial species. **26 – 29**, **32 & 33**, tetraploid species. **35 – 39**, hexaploid species.

The nomenclature for bands used in the present study differs from those used in the previously published studies because of improved resolution. Wild *H. annuus* forms (11-14) and other diploid annual species (not shown) had the majority of bands present in cultivated *H. annuus.* Accessions of wild diploid perennial species (15-20 & 22-25) differed from annual species in

the absence of inhibitors with positions “c-f”, confirming the significant evolutionary distance between the species groups possessing genomes A and B. The majority of tetraploid species listed in the legend to Fig.1 A also lacked these bands. Only one of two *H. hirsutus* accessions had band “d” (Fig. 1 B, 27) while *H. strumosus* L. had a band at position “g” not present in *H. annuus* (32). In hexaploid species, bands “c & f” were present in accession of *H. resinosus* Small (37). *H. multiflorus* L. had bands “e & f” (38) and *H. tuberosus* had a weak band “e”. (39). The presence of bands “c-f” in polyploids can be the result of cross-pollination or the combination of different genomes. The absence of some seed proteins from diploid perennial species of *Helianthus* in comparison with annual species were described earlier (Anisimova, 1996). Purified T/SI components (Fig. 2, T, 17 and S, 1) had mobility on TLGF between aprotinin (M_r 6500) and cytochrome c (M_r 14400). Component “h” of *H. annuus* had a higher molecular weight than other T/SIs (Konarev *et al*., 2000). Some of T/SI components are also present in leaves and heads of *H. annuus.* T/SI were found to inhibit extracellular subtilisin-like proteinases of the white rot fungus *Sclerotinia sclerotiorum*, an important pathogen of sunflower, indicating a possible protective role (Konarev, *et al*., 1999a).

4. Proteinase inhibitors in *Compositae*

Typical representatives of three main *Compositae* subfamilies were analyzed for inhibitors of trypsin and subtilisin (Fig. 2 & 3, Tab.1).

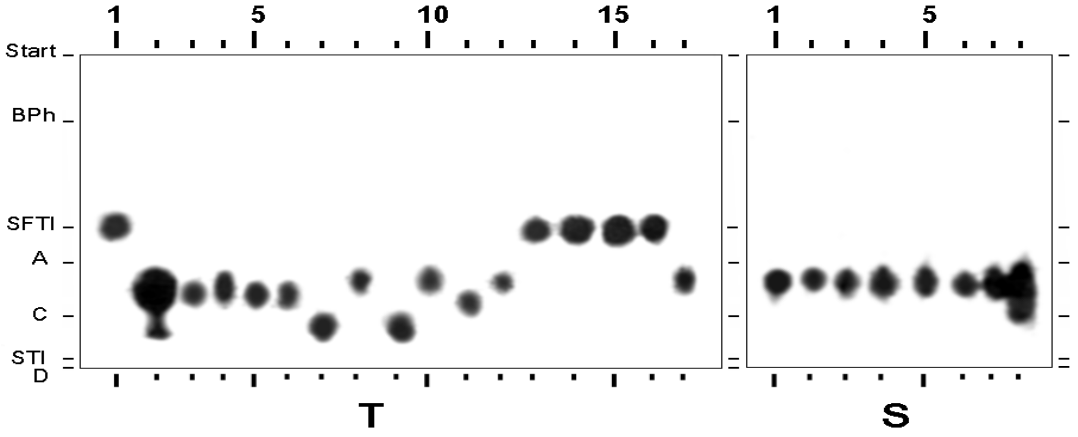

Fig.2. Variation of native proteinase inhibitors of *Compositae* species separated by molecular mass. Water-soluble seed proteins were loaded on gels in volumes of 0.5-2 μl and separated by thin layer gel filtration in Sephadex G-50 (superfine). Gelatin replicas from gels were developed using trypsin (T) and subtilisin (S). Positions of markers; BPh, bromphenol blue (M_r 692); SFTI, main sunflower TI (M_r 1501); A, TI from bovine lung (M_r 6500); C, cytochrome c (M_r 12400, path 7.8 cm); STI, soybean TI (M_r 21000); D, Dextran Blue (path 9 cm). **T**. **1 & 13**, *H. annuus* VIR-104; **2**, *Dahlia pinnata*; **3**, *Silphium perfoliatum*; **4**, *Cosmos bipinnatus*; **5**, *Zinnia* elegans; **6**, *Rudbeckia laciniata*; **7**, *Gailardia* aristata; **8**, *Bidens pilosa*; **9**, *Eclipta prostrata*; **10**, *Senecio viscosus*; **11**, *Solidago virgaurea*; **12**, *Centaurea cyanus*; **14**, *H. tuberosus*; **15**, *Tithonia diversifolia*; **16 & 17**, *H. annuus* inhibitors purified by affinity chromatography and HPLC: **16**, SFTI; **17**, fraction T/SI. **S**. **1**, *H. annuus*, fraction T/SI; **2**, *Carthamus tinctorius*; **3**, *Centaurea cyanus*; **4**, *C. triumfettii*; **5**, *Lactuca sativa*; **6**, *Taraxacum officinale*; **7**, *Senecio viscosus*; **8**, *Cosmos bipinnatus*.

Subfamily *Carduoideae.* All studied accessions of safflower, an important oilseed crop, representing one cultivated species (*Carthamus tinctorius* L, 21 accessions of different origin) and four wild species (*C. oxyacantha* Bieb., *C. lanatus* L., *C. glaucus* Bieb. *and C. palaestinus* Eig) had only one T/SI band with an isoelectric point (pI) about 7.0 as shown in Fig. 3, 1-3. No variability in the inhibitors of this genus was found. Seeds of some *Centaurea* L. species (*C. cyanus* L. and *C. triumfettii* All.) also contained mainly T/SI but differed in their IEF spectra (Fig.3, 6 & 7). Seed of *C. scabiosa* L. (4) had only subtilisin inhibitor (SI) and *C. jacea* L. (not shown) had no inhibitors. The differences in inhibitor spectra are in good agreement with new views on the taxonomy of the subtribe *Centaureinae* Dumort. (tribe *Cardueae* Cass.) including *Cardueae* species (Wagenitz & Hellwig, 1996). Leaves of *C. scabiosa* (5) contained T/SI and SI. Seeds of *Cirsium* L., *Carduus* L.and *Serratula* L.

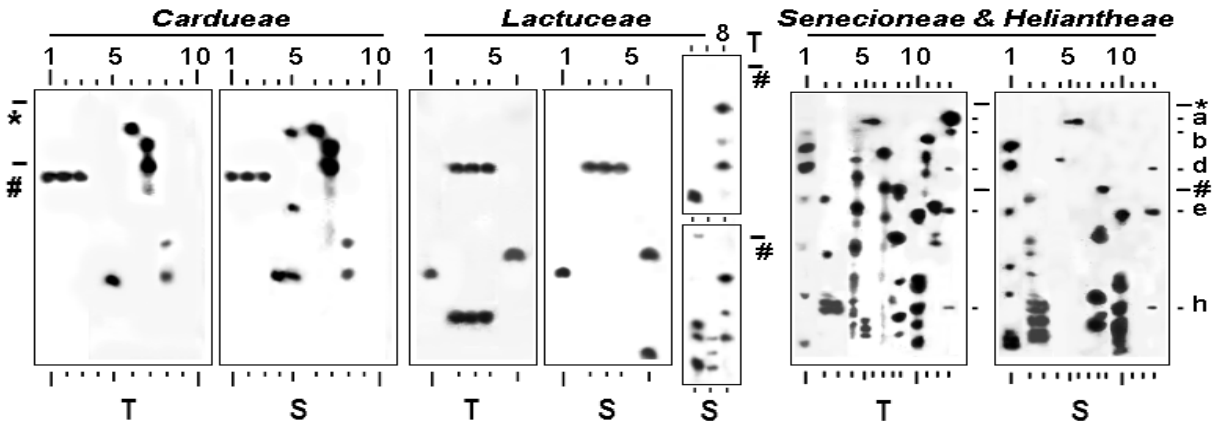

Fig. 3. Polymorphism of proteinase inhibitors in seeds of representatives of tribes ***Cardueae* Cass., *Lactuceae* Cass**., ***Senecioneae* Cass.** and ***Heliantheae* Cass**. Water soluble seed proteins were loaded on gels in volumes of 2 µl for *Cardueae* and *Lactuceae* and 0.3-1 µl for the others and separated by IEF in the pH range 3-10 (for *Lactuceae*, slots 6-8, the pH range was 5-8) with distance between electrodes 5 cm for *Cardueae* and *Lactuceae* and 11 cm for the rest. Inhibitors were detected in gelatin replicas obtained from gels and developed with trypsin (T) and subtilisin (S). **"#" & "*"**, positions of pI markers (pI 7.3 & 10.6). **a-h**, positions of *H. annuus* inhibitor bands. ***Cardueae***. **1- 3**, *Carthamus* sp.: *C. tinctorius*, *C. oxyacantha* & *C. lanatus*; **4 – 7**, *Centaurea* sp.: **4 & 5**, *C. scabiosa*, seed and leaf; **6**, *C. cyanus*; **7**, *C. triumfettii*. **8**, *Cirsium arvense*; **9**, *Arctium tomentosum*; **10**, *Cousinia badghysi*. ***Lactuceae***. **1**, *Sonchus arvensis*; **2 – 4**, *Lactuca* L.: **2**, *L. sativa*; **3**, *L. serriola*; **4**, *L. livida.* **5**, *Cichorium intybus*; **6 – 8**, *Taraxacum* L.: **6**, *T. officinale*; **7**, *T. kok-saghyz*; **8**, *T. hybernum*. ***Senecioneae* ,1**, *Senecio viscosus*. ***Heliantheae*. 2**, *Bidens pilosa*; **3**, *B. radiata*; **4**, *Gailardia aristata* ; **5**, *Rudbeckia laciniata*; **6**, *R. speciosa*; **7**, *Zinnia* elegans; **8**, *Cosmos bipinnatus*; **9**. *C. caudatus*; **10**, *Dahlia pinnata*; **11**, *Silphium perfoliatum*; **12**, *Eclipta prostrata*; **13**, *H. annuus* VIR-104.

species contained weak T/SI. Seeds of species (sp.) of *Arctium* L. and the related genera *Cousinia* Cass. (5 sp.) and *Saussurea* DC. (2 sp.) did not contain any inhibitors.

Table 1. Distribution of proteinase inhibitors in *Compositae*

Subfamily, **tribe**, subribe, genus, species	T/SI	TI	SI
Carduoideae	+/-	-	+/-
Centaureinae Dumort.	+/-	-	+/-
Carthamus L.	+	-	-
Arctium L.	-	-	-
***Cichorioideae*, *Lactuceae* Cass.**	+/-	+/-	+/-
Asteroideae	+/-	+/-	+/-
***Astereae* Cass.** (5 genera)	+/-	+/-	-
Solidago virgaurea L.	+!	-	-
Erigeron uniflorus L.	-	+	-
***Senecioneae* Cass.,** *Doronicum* , *Emilia* Cass., *Ligularia* Cass., *Erechtites* Rafin., *Senecio* L.	+/-	-	+
Senecio viscosus L.	+!	-	-
***Inuleae* Cass.,** *Helichrysum* Mill., *Inula* L.	+/-	-	-
***Tageteae* Cass.,** *Tagetes* L.	-/w?	-	-
***Eupatorieae* Cass.,** *Ageratum* L., *Eupatorium* L.	+/-	-	+/-
***Anthemideae* Cass**. (7 genera)	-	+/-	-
Chrysanthemum L.	-	-	-
Achillea millifolium L.	-	+	-
***Calenduleae* Cass.,** *Calendula* L. *Dimorphotheca* Moenh.	-	+	+

Subfamily, **tribe**, subribe, genus, species	T/SI	TI	SI
***Heliantheae* Cass.** (18 genera)	+/-	+/-	+/-
Echinacea purpurea L.	-	+	+
Arnica iljinii (Maguire) Iljin	+	-	+
Bidens pilosa L.	+!	-	+
Gailardia aristata Pursh	+	+!	-
Rudbeckia laciniata L.	+	+!	+
Rudbeckia speciosa Wend.	+	-	+LpI
Zinnia elegans Jacq.	+w	+!	-
Coreopsis L.	+	-	-
Cosmos bipinnatus Cav.	+!	-	+!
Cosmos caudatus Kunth	+	-	+
Dahlia pinnata Cav.	+!	-	-
Silphium perfoliatum L.	-	+!	-
Eclipta prostrata L.	-	+!	+w
Wedelia Jacq., *Galinsoga* Ruiz. et Pav.	-/w?	-/w?	-/w?
Helianthinae Dumort.	+/-	+/-	+/-
Helianthus L.	+	+H!	-
Tithonia diversifolia	+	+H!	-
Simsia Pers. , *Enceliopsis* A.Nels., *Alvordia* T.S.Brandeg., *Florencia* DC.,	-/w?	-/w?	-/w?

"+" or "-", presence or absence of inhibitors; "+/-", variability inside taxon by presence/absence of inhibitors; "!", high inhibitor activity in seeds (volume of extract less than 0.4 µl is enough for clear IEF spectra); -/w?, extremly low or absent inhibitor activity (5µl of extract is not enough for obtaining clear spectrum); LpI, pI lower than range of Fig.1 & "H", presence of TI type of SFTI.

Subfamily *Cichorioideae*, tribe *Lactuceae* Cass. *Lactuca sativa* L. (23 var.), *L. serriola* L., *L. livida* Boiss. et Reut. and *L. quercina* L. had T/SI with pI about 7.3 and TI with low pI as shown in Fig. 3, slots 2-4, without any variation. Three *Taraxacum* L. species, including two rubber plants, had species-specific T/SI and SI spectra (6-8). Some species of six other studied genera contained T/SI, TI or SI or lacked inhibitors.

Subfamily *Asteroideae*. Analysis of 37 genera from 8 tribes showed that the least active inhibitors are characteristic of seeds of tribes *Anthemideae* Cass. (*Achillea* L., *Artemisia* L., *Chrysanthemum* L., *Leucanthemum* Hill, *Matricaria* L., *Pyrethrum* Zinn, *Tanacetum* L., *Tripleurospermum* Sch.Bip.) and *Tageteae* Cass. The most active, variable and heterogeneous inhibitors were found in species of tribe *Heliantheae* Cass. Results for some accessions are shown in Fig. 3 and Table 1.

As a rule, when several species of the same *Asteroideae* genus were analyzed, IEF spectra of inhibitors were species-specific (*Bidens* L., *Cosmos* Cav., *Rudbeckia* L. etc.). If the set of inhibitor types of *Cardueae* can be considered as minimal (T/SI mainly), the inhibitors of *Asteroideae* and, especially *Heliantheae* are most diverse. T/SI are the most widely distributed inhibitor type in the *Compositae.* Comparison of native inhibitors on the basis of M_r (Fig. 2) showed similarity of T/SIs in representatives of main *Compositae* groups, including *Centaurea* or *Helianthus.* Of course, TLGF gives only approximate estimates of M_r and further researches are necessary to confirm the level of similarity of various T/SIs. Inhibitors of the type SFTI are present only in *Helianthus* and *Tithonia* Desf. ex Gmelin species and absent even from close relatives from the subtribe *Helianthinae.* TIs in other *Heliantheae* groups (*Gailardia* Foug. , *Zinnia* L., *Silphium* L. etc.) had much higher M_r than SFTI.

Conclusions

Seeds of *Helianthus* and other species of *Compositae* contain diverse proteinase inhibitors. The level of taxonomic specificity of inhibitor spectra varies from lines to species or groups of species. Leaves of *Compositae* also contain specific inhibitor systems. Simple approaches to the analysis of inhibitors can be used to study their occurrence in relation to roles as defence proteins or as genetic markers and to identify novel inhibitor types. Further studies of inhibitors of various species of the *Compositae* are in progress.

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References

Anisimova I.N. (1996). Seed proteins as markers in *Helianthus*. In D.J.N.Hind & H.J.Bentje (eds). Compositae: Biology and Utilization. Proceedings of the International Compositae Conference, Kew, 1994. Royal Botanic Gardens, Kew. V.2, 627-641

Bremer K. (1996). Major clades and grades of the *Asteraceae*. In D.J.N.Hind & H.J.Bentje (eds). Compositae: Systematics. Proceedings of the International Compositae Conference, Kew. Royal Botanic Gardens, Kew. 1994. V.1, 1-7

Kollipara, K.P. and Hymowitz, T. (1992). Characterization of trypsin and chymotrypsin inhibitors in the wild perennial *Glycine* species, *J. Agric. Food. Chem*. 40, 2356-2363.

Konarev, Al.V. (1982). Component composition and genetic control of insect -amylase inhibitors from wheat and *Aegilops* grain, *Doklady Vaskhil* 6 (1982), 42-44 (Translated in English by Allerton Press as "Soviet Agricultural Science")

Konarev, Al.V. (1996) Interaction of insect digestive enzymes with plant protein inhibitors and host parasite co-evolution, *Euphytica* 92, 89-94.

Konarev, Al.V. (1986). Analysis of protease inhibitors from wheat grain by gelatine replicas method, *Biochemistry (Moscow)* 51, 195-201. (Translated in English by Plenum Publishing Corp.)

Konarev, Al.V., Kochetkov, V.V., Bailey, J.A., and Shewry, P.R. (1999a): The detection of inhibitors of the *Sclerotinia sclerotiorum* (Lib) de Bary extracellular proteinases in sunflower, *J. Phytopathology* 147, 105-108.

Konarev Al.V., Anisimova I.N., Gavrilova V.A., Shewry P.R. (1999b). Polymorphism of inhibitors of hydrolytic enzymes present in cereal and sunflower seeds. In: Genetics and Breeding for Crop Quality and Resistance. Ed. by G.T. S. Mugnozza, E. Porceddu and M.A. Pagnotta. Kluwer Acad.Publ. 135-144.

Konarev, Al.V., Anisimova, I.N., Gavrilova, V.A., Rozhkova, V.T., Fido, R., Tatham, A.S., and Shewry, P.R. (2000). Novel Proteinase Inhibitors in Seeds of Sunflower (*Helianthus annuus* L.): Polymorphism, Inheritance and Properties. Theoretical and Applied Genetics, 100(1), 82-88

Kumar G.N., Houtz R.L., and Knowles N.R. (1999) Age-induced protein modifications and increased proteolysis in potato seed-tubers Plant Physiol. 119, 89-100.

Luckett S., Garcia R.S., Barker J.J., Konarev A.V., Shewry P.R., Clarke A.R., Brady R.L. (1999) High-resolution structure of a potent, cyclic proteinase inhibitor from sunflower seeds. J Mol Biol 1999, Jul 9; 290(2):525-33

Ryan, C.A., (1990). Protease inhibitors in plants: gens for improving defenses against insects and pathogens, *Annual. Rev. Phytopathol.* 28, 425-449.

Shewry, P.R., (1999). Enzyme inhibitors of seeds: types and properties. In P.R. Shewry & R. Casey (eds) Seed Proteins. Kluwer Academic Publishers, The Netherlands, 587-615.

Shewry, P.R., and Lucas J.A. (1997). Plant proteins that confer resistance to pests and pathogens, *Adv. Bot. Res*. 26 135-192.

Wagenitz G. & Hellwig F.H. (1996). Evolution of characters and phylogeny of the *Centaureinae*. In D.J.N.Hind & H.J.Bentje (eds). Compositae: Systematics. Proceedings of the International Compositae Conference, Kew, 1994. Royal Botanic Gardens, Kew.V.1, 491-510.