Antinutritional Factors in Faba Beans (*Vicia faba* L.) As Affected by Breeding toward the Absence of Condensed Tannins

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The effect of genetic removal of condensed tannins on the levels of other antinutritional factors in faba beans (*Vicia faba* L.) has been studied by comparison between partners of six near-isogenic pairs for trypsin inhibitor activities (TIA), concentrations of lectins and pyrimidine glucosides (vicine and convicine), seed weight, and the proportion of testa thereof. Each near-isogenic pair consisted of two lines which were genetically almost identical, apart from the absence [tannin-free (TF)] or presence [tannin-containing (TC)] of condensed tannins. Within these pairs no effect of tannin content was observed on cotyledon-bound TIA or lectin concentrations. Seeds of the TF lines showed higher levels of pyrimidine glucosides, lower seed weight, and lower proportion of testa as compared to the TC lines of the same near-isogenic pair. Methanolic extracts from testa of exclusively the TC lines showed significant TIA with both porcine and bovine trypsin. This TIA was completely inhibited by 1% poly(vinylpyrrolidone). These data indicate that condensed tannins are responsible for the testa-bound TIA. The observations are discussed in relation to the consequences of breeding faba beans, free of condensed tannins, for the agricultural characteristics and for the nutritional quality of the crop.

INTRODUCTION

Several legume species show high protein levels in seeds and foliage and are therefore useful as fodder crops. Besides protein, legumes also contain antinutritional factors (ANFs) which negatively affect the digestibility. In seeds of *Vicia faba* L. (faba bean) four ANF types are observed: (1) condensed tannins, which are located in the testa; (2) trypsin inhibitor activity (TIA); (3) lectins; (4) pyrimidine glucosides (vicine and convicine), which accumulate in the cotyledons (Liener, 1989a). The testa themselves, being high in poorly digestible fiber, hardly contribute to the nutritive quality of the bean.

Condensed tannins are considered the major ANF in faba beans, although their antinutritive activity is not yet unequivocally established for all animal species as will be discussed later on. The antinutritive activity may be due to complexation with digestible protein in general and with digestive enzymes, such as trypsin and chymotrypsin, in particular (Liener, 1989a). Tannin-free faba beans can be readily obtained by selection; the lack of tannin is a monogenic recessive trait which can be easily selected for since it is associated with a white color of the flowers (Bond, 1976; Cabrera and Martin, 1989). Because of this association, it cannot be excluded that also other processes, related to phenolic metabolism, are different in white-flowered cultivars of *V. faba*. It is not known whether selection for the absence of condensed tannins leads to an increase or a decrease of the other ANFs in faba beans, e.g., via epistatic or pleiotropic interactions between the biosynthetic pathways.

In this study we investigate the effects of selection for the absence of condensed tannins on the levels of other ANFs by comparison between partners of six near-isogenic pairs, which are distinguishable within each pair by the presence or absence of condensed tannins. The use of more than one near-isogenic pair virtually eliminated the possibility that differences, observed between two partners of one pair, are due to genes distinct from but closely linked to the gene responsible for the synthesis of condensed tannins. Such genes might remain linked to the "tannin genes" during the production of a near-isogenic pair.

MATERIALS AND METHODS

Plant Material, Near-Isogenic Pairs. Pairs of near-isogenic lines of *V. faba* L. were derived from crosses between a white-flowered, tannin-free female parent and a colored-flowered, tannin-containing male parent. The tannin-free parental genotypes Metissa (M), Ewir (E), and Rowena × Minica.M5 (R) were pollinated with Herz Freya (H), Pavane (P), or Hjan Mikko (HM) (Table I), while the tannin-free genotypes Mansholt's wierboon (Ma) and Staygreen (S) were open pollinated with unknown tannin-containing varieties. Heterozygous plants were self-fertilized for six generation cycles, in each of which a single heterozygous progeny plant was used to produce seed for the subsequent generation. After these six cycles of selfing, one homozygous tannin-free plant and one homozygous tannin-containing plant were selected as parents and self-pollinated to produce a pair of near-isogenic lines.

Chemical Analyses. Analyses were performed in four replications of five seeds for each line. Testa and cotyledons were weighed and analyzed separately after the seeds were fractured with a hammer.

Condensed tannins were quantified with the vanillin–sulfuric acid assay according to the method of Kuhla and Ebmeier (1981), modified by using catechin instead of phloroglucinol as a reference compound. Briefly, 200 mg of ground testa tissue (sieve mill 1.0 mm) was extracted in 30 mL of dimethylformamide (80% in water) containing 0.01 M sodium sulfite under reflux conditions for 30 min. Aliquots of 0.5 mL, containing ca. 10 μg of catechin equivalents of condensed tannin, were incubated for 20 min at room temperature in 0.2% vanillin/70% sulfuric acid (w/w) before absorption measurement at 500 nm. For measurement of cotyledon-bound trypsin inhibitor activity (TIA), cotyledons were ground with a mortar and pestle and 100 mg of the produced powder was further homogenized in 10 mL of 0.001 M HCl with an all-glass Potter Elvehjem tissue grinder. Further extraction and TIA analyses were carried out as described by Valdebouze et al. (1980), modified with respect to the initiation of the TIA assay; i.e., trypsin was added as the last component to the inhibitor–substrate mixture (Liu and Markakis, 1989). For TIA measurement in testa, 100 mg of ground tissue (sieve mill, 1.0 mm) was extracted with 10 mL of 70% aqueous methanol. Aliquots of 50, 100, or 200 μL (maximally 100 μg of tannin) from these extracts were used for TIA determination as described above. Vicine and convicine were extracted from 200 mg of mortar-ground cotyledon tissue after homogenization with an all-glass Potter tissue grinder in 10 mL of 5% perchloric acid

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Table I. Tannin Concentration, Weight, and Proportion of Testa in Seeds of Six Near-Isogenic Pairs of *V. faba* L.[a]

near-isogenic pair	±	tannin concn, mg/g of seed	seed wt, g	proportion of testa, % of seed wt
M × H	−	nd	0.58 ± 0.04	11.9 ± 0.1
	+	4.9 ± 0.4	0.66 ± 0.03	14.6 ± 0.2
M × P	−	nd	0.70 ± 0.13	12.2 ± 0.1
	+	7.0 ± 0.2	0.77 ± 0.01	15.7 ± 0.5
E × H	−	nd	0.59 ± 0.04	12.2 ± 0.3
	+	7.4 ± 0.1	0.64 ± 0.03	15.1 ± 0.4
R × HM	−	nd	0.51 ± 0.01	12.2 ± 0.2
	+	6.4 ± 0.5	0.54 ± 0.02	15.7 ± 0.5
Ma, open pollin	−	nd	1.03 ± 0.05	11.3 ± 0.3
	+	6.7 ± 0.5	1.11 ± 0.05	13.4 ± 0.3
S, open pollin	−	nd	0.93 ± 0.06	11.6 ± 0.6
	+	5.0 ± 0.2	1.12 ± 0.04	13.4 ± 0.4

[a] Within each pair the two lines are distinguishable by the presence or absence of condensed tannins. Genotypes used as parents in the production of the near-isogenic pairs: (tannin-free parents) Metissa (M), Ewir (E), Rowena × Minica.M5 (R), Mansholt's wierboon (Ma), Staygreen (S); (tannin-containing parents) Herz Freya (H), Pavane (P), Hjan Mikko (HM); open pollin, open pollinated; −, white-flowered, tannin-free partner line; +, colored-flowered, tannin-containing partner line. Tannin is quantified with catechin as a standard. Results are expressed as mean ± SEM of four replicates with five seeds each. nd, not detectable; detection limit is 0.5 mg/g of seed.

(Marquardt and Frohlich, 1981), containing 6 mg of uridine/mL as an internal standard. After centrifugation and filtration over a 0.22-μm cellulose acetate filter, the three compounds were separated by reversed-phase HPLC, using a Spherisorb ODS 2 C_{18} column (125 × 4 mm, 3-μm particle size) as stationary phase and 0.05 M sodium citrate (pH 6.8) as mobile phase at 1.0 mL/min. Convicine, vicine, and uridine eluted at ca. 2.5, 3.5, and 5 min, respectively, and were quantified by UV absorption at 265 nm. Lectins in faba bean cotyledon tissue were measured by a functional lectin immunoassay (FLIA) using thyroglobulin as a coating in the ELISA (Hamer et al., 1989).

RESULTS

Tannin Content, Seed Weight, and Proportion of Testa. Table I shows that condensed tannins are virtually absent in white-flowered partners of the near-isogenic pairs (detection limit is 0.05% of total seed). The tannin content of the colored-flowered partner lines varies from 4.9 to 7.4 mg/g of seed. White-flowered and colored-flowered partner lines are further referred to as TF (tannin-free) and TC (tannin-containing) lines, respectively.

Seeds from the TF lines showed an up to 20% lower weight and a maximally 30% lower proportion of the poorly digestible testa as compared to the TC line of the same near-isogenic pair (Table I).

Trypsin Inhibitor Activity. *Cotyledon-Bound Trypsin Inhibitor Activity.* Trypsin inhibitor activities (TIA) in aqueous 0.001 M HCl extracts from cotyledon tissue are slightly higher toward bovine trypsin as compared to porcine trypsin (Table II). Cotyledon-bound TIAs range from 0.5 to 1.8 mg of porcine trypsin inhibited/g of tissue and from 0.8 to 2.5 mg of bovine trypsin inhibited/g of tissue. The presence of 1% poly(vinylpyrrolidone) (w/v) in the incubation mixture had no effect (data not shown). With both porcine and bovine trypsin the cotyledon-bound TIAs in the TF line are in general not significantly different from those in the TC line of the near-isogenic pair. Exceptions were found for bovine and porcine trypsin: the TF line of the near-isogenic pair M × P showed a higher TIA than the TC line, while the opposite was observed with porcine trypsin in the near-isogenic pair E × H.

Testa-Bound Trypsin Inhibitor Activity. Methanol extracts from testa of the near-isogenic TC partner lines show TIA (Tables II and III). Pure methanol did not show a significant TIA. In contrast to cotyledon-bound TIA, this TIA in the testa is completely inhibited by 1% poly(vinylpyrrolidone). Such activity is absent in methanol extracts from testa of the TF partner lines. Testa-bound TIA in TC lines increased linearly with the tannin concentration in the incubation mixture up to approximately 50 μg/4.5 mL; the specific activity (=TIA, relative to vanillin reactivity) decreases at higher concentrations (Figure 1). It is up to 1.7 times more active toward porcine trypsin than to bovine trypsin (Table III). The testa-bound TIA per unit of testa weight ranges from almost equal to maximally 7 times higher than the cotyledon-bound TIA per unit of cotyledon weight of the same TC genotype. There are significant differences between the levels of testa-bound TIA (TIA per gram of tissue) of the different TC lines, particularly when porcine trypsin is used as enzyme source. With this trypsin type the TC line from the M × P cross shows the lowest TIA, while the highest specific activity is observed in testa from the TC line of the open-pollinated Ma cross. These differences in levels of testa-bound TIA can only partly be explained by variation in tannin levels between TC lines (Table I); when testa-bound TIA is expressed per unit of tannin weight (Table III), differences between genotypes are still observed for both trypsin types.

Lectins and Pyrimidine Glucosides. Except for the near-isogenic pair derived from the open-pollinated genotype Staygreen, lectin concentrations are not significantly different for TC and TF partners of the same near-isogenic

Table II. Trypsin Inhibitor Activities in Cotyledons and in Testa of Six Near-Isogenic Pairs of *V. faba* L.[a]

		trypsin inhibitor activity			
		cotyledons		testa	
near-isogenic pair	±	porcine trypsin	bovine trypsin	porcine trypsin	bovine trypsin
M × H	−	1.8 ± 0.1	2.3 ± 0.2	−0.3 ± 0.3	−0.1 ± 0.2
	+	1.8 ± 0.1	2.5 ± 0.2	2.7 ± 0.2	2.7 ± 0.3
M × P	−	1.2 ± 0.1	1.6 ± 0.2	0.0 ± 0.3	0.4 ± 0.5
	+	0.8 ± 0.1	1.1 ± 0.2	2.4 ± 0.3	2.2 ± 0.5
E × H	−	0.8 ± 0.1	1.4 ± 0.6	0.5 ± 0.1	0.1 ± 0.3
	+	0.8 ± 0.1	2.2 ± 0.1	3.4 ± 0.2	2.1 ± 0.5
R × HM	−	1.2 ± 0.1	1.6 ± 0.2	0.3 ± 0.1	0.6 ± 0.2
	+	1.2 ± 0.1	1.8 ± 0.1	2.9 ± 0.4	1.8 ± 0.4
Ma, open pollin	−	0.5 ± 0.1	0.8 ± 0.1	0.2 ± 0.3	0.2 ± 0.2
	+	0.6 ± 0.1	0.9 ± 0.2	4.4 ± 0.5	2.5 ± 0.3
S, open pollin	−	1.4 ± 0.2	2.4 ± 0.4	0.4 ± 0.1	0.3 ± 0.3
	+	1.4 ± 0.2	2.3 ± 0.4	2.9 ± 0.4	1.8 ± 0.2

[a] Results are expressed as milligrams of trypsin inhibited per gram of tissue (cotyledon or testa). Cotyledon-bound TIA is measured in the presence of 1% poly(vinylpyrrolidone) and testa-bound TIA in its absence with both porcine and bovine trypsin as enzyme sources. Testa-bound TIA was determined in the presence of maximally 15 μg of tannin in the incubation mixture. For further details and legend to abbrevations, see Table I.

Table III. Testa-Bound Trypsin Inhibitor Activity in the Tannin-Containing Partners of Six Near-Isogenic Pairs of *V. faba* L.[a]

	trypsin inhibitor activity	
near-isogenic pair	porcine trypsin	bovine trypsin
M × H	75 ± 2	61 ± 8
M × P	48 ± 5	41 ± 10
E × H	72 ± 13	42 ± 14
R × HM	76 ± 15	45 ± 10
Ma, open pollin	67 ± 12	56 ± 5
S, open pollin	84 ± 9	51 ± 6

[a] Results are expressed as milligrams of trypsin inhibited per gram of tannin (= catechin equivalent). TIA assays are carried out in the absence of poly(vinylpyrrolidone) with porcine and bovine trypsin with up to 15 μg of tannin in the incubation mixture. For further details and legend to abbreviations, see Table I.

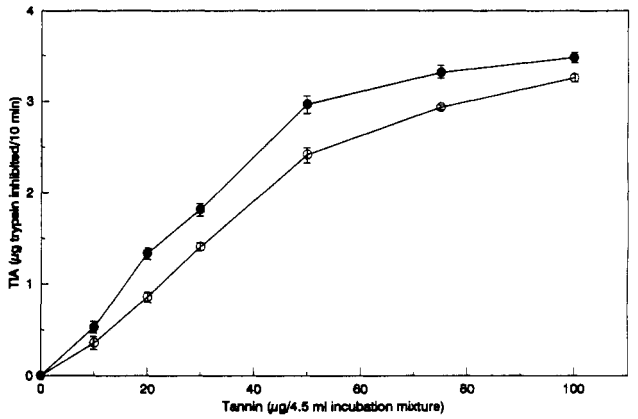

Figure 1. Effect of tannin concentration (in µg/4.5 mL of incubation mixture) on the trypsin inhibitor activity (TIA) toward porcine (●) and bovine trypsin (○). Tannins were isolated from the tannin-containing partner of a near-isogenic pair derived from a cross between the genotypes Ewir and Herz Freya. Tannin is quantified with catechin as a standard. Bars indicate the standard deviations in a triplicate experiment.

Table IV. Levels of Lectins and Pyrimidine Glucosides (Vicine and Convicine) in Cotyledons of Six Near-Isogenic Pairs of *V. faba* L.[a]

near-isogenic pair	±	lectins	vicine	convicine	vicine plus convicine
M × H	−	2.3 ± 0.6	8.6 ± 0.5	2.5 ± 0.3	11.2 ± 0.7
	+	2.4 ± 0.5	7.1 ± 0.3	2.1 ± 0.3	9.2 ± 0.3
M × P	−	2.0 ± 0.3	10.9 ± 0.7	4.5 ± 0.4	15.4 ± 1.0
	+	2.2 ± 0.2	10.1 ± 0.4	3.8 ± 0.2	13.9 ± 0.6
E × H	−	1.5 ± 0.2	11.7 ± 0.9	2.4 ± 0.9	14.1 ± 0.9
	+	1.6 ± 0.2	10.2 ± 0.3	2.5 ± 0.2	12.7 ± 0.3
R × HM	−	1.2 ± 0.2	5.8 ± 0.3	5.8 ± 0.4	11.6 ± 0.7
	+	1.2 ± 0.2	4.3 ± 0.2	5.0 ± 0.2	9.4 ± 0.3
Ma, open pollin	−	2.5 ± 0.4	7.2 ± 0.2	4.1 ± 0.1	11.2 ± 0.2
	+	2.5 ± 0.5	7.0 ± 0.3	3.9 ± 0.1	11.0 ± 0.3
S, open pollin	−	2.3 ± 0.1	8.7 ± 0.6	1.7 ± 0.1	10.4 ± 0.7
	+	1.9 ± 0.1	7.0 ± 0.4	1.5 ± 0.1	8.4 ± 0.5
LSD 5%		0.22	0.27	0.13	0.66

[a] Results are expressed in milligrams per gram of cotyledon tissue. For further details and abbreviations, see Table I.

pair (Table IV). Between the six different near-isogenic pairs the lectin concentrations vary by a factor of 2, and the differences are significant at $P < 0.05$ between some of these pairs.

Levels of the combined pyrimidine glucosides, vicine and convicine, are significantly higher in the cotyledons of the TF partners as compared to the TC partners of the same near-isogenic pair (Table IV, $P < 0.001$). Analysis of variance also showed significant differences between the six near-isogenic pairs (Table IV, $P < 0.05$).

DISCUSSION

The results in this study show that selection for the absence of condensed tannins is associated with a lower proportion of testa of the seed, a lower seed weight, the absence of testa-bound trypsin inhibitor activity, and a higher level of pyrimidine glucosides in the cotyledons. Genetic removal of tannins had no effect on cotyledon-bound levels of trypsin inhibitor activity or lectins. The testa-bound TIA is probably directly related to the presence of condensed tannins (see below). It is, however, not clear from this study whether the effects of selection for the absence of tannins on the other ANFs are epistatic or pleiotropic. The association between the absence of condensed tannins with a higher level of pyrimidine glucosides through activity of a single gene, a pleiotropic effect, seems to be unlikely since the biosynthetic pathways of both ANF types are not related.

The genetic variation observed for ANFs in this study are in agreement with earlier studies on condensed tannins (Kuhla et al., 1982), lectins (Marquardt et al., 1975; Van der Poel et al., 1992), cotyledon-bound trypsin inhibitor activity (Marquardt et al., 1975; Valdebouze et al., 1980; Wiseman et al., 1991), and pyrimidine glucosides (Duc et al., 1989; Marquardt, 1989; Wang et al., 1990). In addition, Duc et al. (1989) described a mutant of *V. faba* L. that is virtually free of pyrimidine glucosides. It should be noted that tannin mass was quantified in the present study with (+)-catechin as a standard. According to Price et al. (1978), this may lead to a slight overestimation of tannin content.

The occurrence of testa-bound trypsin inhibitor activity has never been thoroughly investigated. Liener (1989a) and Valdebouze et al. (1980) mentioned the potential testa-bound TIA caused by condensed tannins. Wiseman et al. (1991), using only one near-isogenic pair, observed by a similar approach as in our study that testa extracts of the tannin-containing partner showed TIA, while extracts from the tannin-free partner did not. The occurrence in each of the six near-isogenic pair used in this study of testa-bound TIA in exclusively tannin-containing partners confirms this observation. The reproducibility in all six pairs virtually excludes the possibility that this TIA is caused by the product of one or more genes, which are linked to, but different from, the tannin gene itself. The suggestive, causal relationship between testa-bound TIA and the presence of condensed tannins is further substantiated by the inactivation of this TIA by poly(vinylpyrrolidone). The differences between TC lines in testa-bound TIA per unit of tannin may be related to differences in composition of the condensed tannins, e.g., in the degree of polymerization of the (+)-catechin and (−)-epicatechin units. The level of testa-bound TIA mostly exceeds that of cotyledon-bound TIA when expressed per unit of tissue from which it was extracted. Since seeds of the TC lines consist of only approximately 12% testa, the cotyledon-bound TIA per seed is still higher.

Indications for a lower proportion of testa tissue in the seed and a lower seed weight in tannin-free as compared to tannin-containing faba beans, as has been shown by comparison for the six TF vs TC partners in our study, have been obtained before (Bond, 1976; Singh and Tomer, 1988). Singh and Tomer (1988) used seed weight and proportion of testa as yield components to explain a lower seed production of tannin-free as compared to tannin-containing genotypes. This assumption was supported by field tests performed in the framework of the European Joint Faba Bean Test (Ebmeyer, personal communication, report available from Dr. E. Ebmeyer, c/o F. von Lochow-Petkus, P.O. Box 1311, D-1311 Bergen, Germany) with two near-isogenic pairs, one of which was the pair derived from open-pollinated Staygreen used in the present study. In these field tests the tannin-free lines showed 5–7% lower seed yields than the tannin-containing partner lines of the same near-isogenic pair. A lower yield of tannin-free faba beans was attributed to a higher susceptibility to diseases such as foot-rot (Van Norel, 1985; Villalobos and Jellis, 1990), although the evidence for a protective role of tannins is controversial. Villalobos and Jellis (1990) found a significantly higher resistance to *Fusarium* foot-rot in the tannin-containing partners of two near-isogenic pairs, while investigations at our laboratory with six other near-isogenic pairs showed no effect of the absence of tannins on resistance to the same disease (Van Loon et al., 1989; Helsper et al., 1991, 1992).

The effects of the genetic removal of tannins from faba beans on the digestibility may differ between animal species in relation to their susceptibility to tannins and to the effects of the individual other ANFs. The presence

of tannins in protein-rich fodders appears to be beneficial for ruminants; the formation of tannin–protein complexes slows down the high digestive activity for protein in these animals and thus prevents bloat (Kendall, 1966; Reid et al., 1974). In piglets and nonruminant animals, tannins may cause negative nutritive effects [for a review, see Marquardt (1989)]. Studies with the near-isogenic line pair, derived from open-pollinated Staygreen, showed a lower ileal and fecal digestibility of nitrogen and dry matter in piglets of the tannin-containing partner as compared to the tannin-free partner (Van der Poel et al., 1992). In chickens, the effect of condensed tannins is controversial; Marquardt (1989) provided evidence for growth inhibition caused by condensed tannins, while Jansman et al. (1989) found no effect. Moreover, in a study with a near-isogenic pair no effect of tannins was found on the apparent metabolizable energy in poultry (Wiseman et al., 1991).

In investigations on the effect of condensed tannins on digestibility other antinutritional factors have to be considered also. As shown in our study with near-isogenic pairs, the impaired nutritive quality of tannin-containing faba beans may be attributed not only to an aspecific binding of digestible protein but also to tannin-related trypsin inhibitor activity and to the higher proportion of poorly digestible testa (Bjerg et al., 1988). On the other hand, when tannins have no antinutritive effect, as is possibly the case in poultry, the genetic removal of tannins may have a negative influence on animal performance by the associated increase in levels of vicine and convicine (Table III) which affect egg production and fertility (Liener, 1989b).

The above considerations demonstrate the necessity to assess the antinutritive activity of each individual antinutritional factor for each different animal species when the beneficial effect of the genetic removal of condensed tannins is to be established.

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