

Evolutionary relationships among proteins in the phytohemagglutinin-arcelin- α -amylase inhibitor family of the common bean and its relatives

T. Erik Mirkov¹, Janice M. Wahlstrom¹, Kiyoshi Hagiwara², Flavio Finardi-Filho^{1,3}, Susanne Kjemtrup¹ and Maarten J. Chrispeels^{1,*}

¹Department of Biology, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0116, USA (* author for correspondence); ²Present address: National Institute of Agrobiological Resources 2-1-2, Kannondai, Tsukuba, Ibaraki, 305 Japan; ³Present address: Departamento de Alimentos e Nutrição Experimental, FCF-University of São Paulo, SP, Brazil

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Abstract

The common bean, *Phaseolus vulgaris*, contains a family of defense proteins that comprises phytohemagglutinin (PHA), arcelin, and α -amylase inhibitor (α AI). Here we report eight new derived amino acid sequences of genes in this family obtained with either the polymerase chain reaction using genomic DNA, or by screening cDNA libraries made with RNA from developing beans. These new sequences are: two α AI sequences and arcelin-4 obtained from a wild accession of *P. vulgaris* that is resistant to the Mexican bean weevil (*Zabrotes subfasciatus*) and the bean weevil (*Acanthoscelides obtectus*); an α AI sequence from the related species *P. acutifolius* (tepary bean); a PHA and an arcelin-like sequence from *P. acutifolius*; an α AI-like sequence from *P. maculatus*; and a PHA sequence from an arcelin-5 type *P. vulgaris*. A dendrogram of 16 sequences shows that they fall into the three identified groups: phytohemagglutinins, arcelins and α AI. A comparison of these derived amino acid sequences indicates that one of the four amino acid residues that is conserved in all legume lectins and is required for carbohydrate binding is absent from all the arcelins; two of the four conserved residues needed for carbohydrate binding are missing from all the α AI. Proteolytic processing at an Asn-Ser site is required for the activation of α AI, and this site is present in all α AI-like sequences; this processing site is also found at the same position in certain arcelins, which are not proteolytically processed. The presence of this site is therefore not sufficient for processing to occur.

Introduction

The common bean, *Phaseolus vulgaris*, contains a family of plant defense proteins that comprises

phytohemagglutinin (PHA), arcelin (ARL), and α -amylase inhibitor (α AI) [5]. The genes for these three proteins are encoded by a single locus in the *P. vulgaris* genome [29] and it is likely that these

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers: U10348 (α AI2 Pvul(w)), U10415 (α AI-like Pacu), U10353 (α AI-like Pmac), U10352 (α AI3 Pvul(w)), U10416 (PHA-L Pacu), U10349 (PHA-E Pvul(w)), U10351 (ARL4 Pvul(w)) and U10350 (ARL Pacu).

homologous genes have arisen by duplication of a single ancestral gene. Derived amino acid sequences obtained from cDNA clones have been determined for PHA [12], α AI (first identified as a lectin-like protein) [13], and arcelin [30]. The polypeptides show considerable (45–85%) amino acid sequence identity and it is therefore predicted that the proteins have similar structures as well [38]. PHA and α AI are found in domesticated bean varieties as well as in wild accessions; arcelin has so far been found only in certain wild accessions of *P. vulgaris* that come from Mexico [31]. The three proteins have different modes of action in protecting seeds from being eaten by mammals or infested by bruchid larvae that burrow into the seeds: PHA is a lectin that binds to glycoproteins in the intestinal mucosa of mammals and insects and its toxicity results from this initial binding (see [36] for review), whereas α AI inhibits the α -amylase in the digestive tract of mammals and coleoptera [32, 33]. The mechanism of action of arcelin remains to be elucidated: it may be toxic [31], or it may simply be indigestible [22].

PHA and arcelin are glycoproteins whose mature deglycosylated polypeptides have molecular weights of 27 000 and 26 200, respectively. α AI is encoded by a polypeptide of similar size, but after proteolytic processing the deglycosylated α and β subunits have molecular weights of respectively 7800 and 14 000 [26, 46].

α AI inhibits porcine pancreatic α -amylase activity by forming a 1:1 complex with the enzyme, and it probably inhibits insect α -amylases in the same way. However, because of the coevolution of pests with their food sources, there is likely to be considerable specificity in the interaction between different α AI and the α -amylases present in different pests, and therefore variability in the α -amylase inhibitors. Thus, α AI from domesticated bean cultivars inhibits the α -amylases of Old World bruchids such as *Callosobruchus maculatus* and *C. chinensis*, although it does not inhibit the α -amylases of the New World bruchid *Zabrotes subfasciatus* [16]. However, wild accessions of *P. vulgaris* contain inhibitors that do inhibit the α -amylases of the New World bruchids

[42]. Bruchids cause considerable losses of legume crops in developing as well as in developed countries, and the genes for α AI are likely vehicles to transfer bruchid resistance from the common bean to other legumes by genetic engineering. We recently succeeded in expressing the bean α AI gene in seeds of pea (*Pisum sativum*) at levels that are sufficiently high to give complete protection against *C. maculatus* and *C. chinensis* [40]. Within the genus *Phaseolus* it is possible to transfer resistance by traditional breeding techniques. Cardona *et al.* [3] succeeded in transferring resistance to *Z. subfasciatus* from resistant wild accessions of *P. vulgaris* to a susceptible domesticated variety using backcrossing. Because selection after each cross was done on the basis of arcelin, the entire PHA- α AI-arcelin locus was probably transferred from the wild accessions. The final backcrossed lines have high levels of arcelin and it is likely that these high levels are responsible for the insect-resistance phenotype. However, α AI may also contribute to the resistance. The same backcrossing strategy did not work to transfer resistance to *A. obtectus*, and it has not been possible to transfer resistance against this bruchid from resistant wild accessions to domesticated varieties [19]. In view of the promising results obtained with gene transfer that allowed the creation of bruchid-resistant legume seeds [40], we consider it important to study this gene family in much greater detail and to examine the biological specificity of the α AI present in wild accessions of *P. vulgaris* and in other species in the genus *Phaseolus*. Here we report the newly derived amino acid sequences of a number of putative α AI, α AI-like proteins, PHAs and arcelins, and discuss their evolutionary relatedness.

Materials and methods

Plant materials

The following species and accessions were used, and the order given is the same as in Fig. 1. Species not listed below or in Fig. 1, but also used in

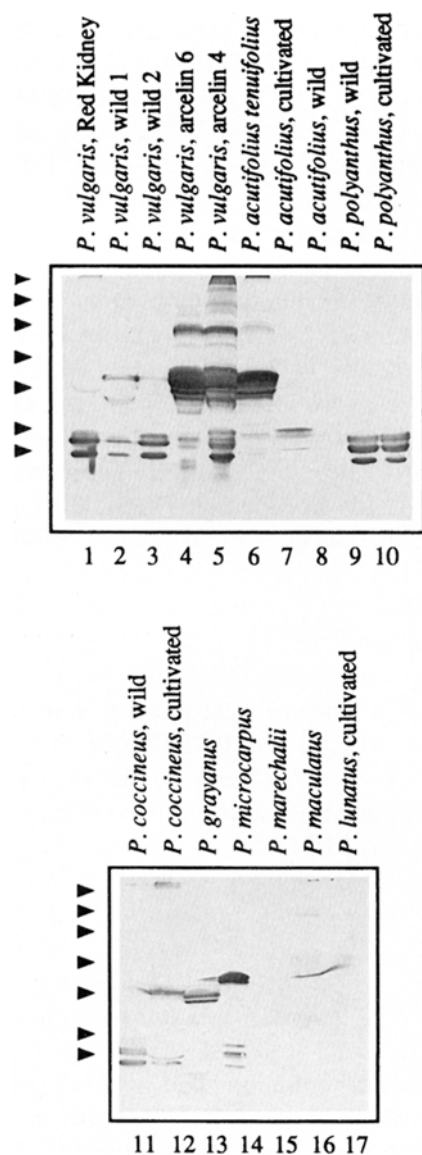


Fig. 1. Immunoblot analysis of the SDS-PAGE separated crude extracts from the seeds numbered 1–17 (as described in the text). Arrows represent the molecular weight markers (from bottom to top: 15 200, 18 600, 27 600, 46 300, 74 700, 105 700, 221 200). Blots were probed with antiserum raised against α AI1. Lanes were loaded for equal proteins.

this study, were *P. vulgaris* cv. Tendergreen and *P. vulgaris*, wild, arcelin-5, G-2771. Species for which the accession numbers are shown were obtained from the Centro Internacional de Agricultura Tropical in Cali, Colombia through the generosity of Rigoberto Hidalgo. Species without

accession numbers were obtained from Native Seeds/Search in Tucson, Arizona. The arcelin type beans were given to us by R. Bollini, CNR, Milano, Italy. The species and accessions involved were: 1, *P. vulgaris* cv. red kidney; 2, *P. vulgaris*, wild, arcelin-5, G2771; 3, *P. vulgaris*, wild, G24408; 4, *P. vulgaris*, wild, arcelin-6, G11051; 5, *P. vulgaris*, wild, arcelin-4, G12949; 6, *P. acutifolius tenuifolius*; 7, *P. acutifolius*, cultivated; 8, *P. acutifolius*, wild, G40102; 9, *P. polyanthus*, wild, G35877; 10, *P. polyanthus*, cultivated, G35532; 11, *P. coccineus*, wild, G35875; 12, *P. coccineus*, cultivated, G35162; 13, *P. grayanus*, wild, S13183; 14, *P. microcarpus*, wild, S13308; 15, *P. marechalii*, wild, S26152; 16, *P. maculatus*, cocolmecha bean; 17, *P. lunatus*, cultivated, G25780.

Protein extraction

Proteins were extracted from seeds by grinding in an ice-cold mortar with a buffer containing 10 mM Tris-HCl pH 7.5, 0.5 M NaCl, 0.1% Triton X-100 and 1% 2-mercaptoethanol supplemented with 1 μ g/ml of leupeptin, aprotinin, and pepstatin A (125 mg of seeds per ml of buffer). The supernatant obtained after centrifugation at $15000 \times g$ for 5 min (twice) was frozen at -20°C until further use.

Immunoblot analysis

For immunoblotting, appropriate quantities of protein (determined according to [21]) were fractionated by SDS-PAGE, transferred to nitrocellulose, and the α AI detected using a rabbit anti- α AI serum obtained as described [26]. We used goat anti-rabbit IgG coupled to horseradish peroxidase (BioRad) as the secondary antibody.

Inhibition tests

Inhibitory activity against porcine pancreatic α -amylase (EC 3.2.1.1.; type IA, Sigma) was

tested by the method of Bernfeld [2] with 0.02 M potassium phosphate pH 7.5, 0.067 M NaCl, 0.1 mM CaCl₂. Seed extracts were pre-incubated with the enzyme for 30 min at 37 °C prior to measuring activity. All tests were carried out with 1 unit of α -amylase, defined as the amount of enzyme required to produce, from starch, the reducing equivalents of 1 μ mol maltose in 10 min at 37 °C.

cDNA library construction and screening

Total RNA was purified from developing beans by the procedure of Dunsmuir *et al.* [9]. Poly(A)⁺ RNA was isolated by oligo(dT)-cellulose chromatography (PolyATtract mRNA Isolation System III, Promega Corporation, Madison, WI). cDNA was synthesized with a *Not*I-oligo(dT) primer-adaptor (Promega) according to the method of Lapeyre and Amalric [20]. cDNAs were made blunt-ended with T4 DNA polymerase before the addition of *Eco*RI adaptors (Promega). After adaptor addition, excess adaptors were removed by Sephacryl S-400 chromatography. cDNAs larger than 0.4 kb were ligated into λ gt10 that had been digested with *Eco*RI and dephosphorylated (Promega). Following packaging (Gigapack II Gold Packaging Kit, Stratagene Cloning Systems, La Jolla, CA), the libraries were screened with the cDNA clone coding for α AI [26]. Selected clones were subcloned in pGEM11Zf(–) (Promega) and sequenced by the Sequenase dideoxy chain termination method (United States Biochemical Corporation, Cleveland, OH).

Genomic DNA preparation and PCR analysis

Genomic DNA was purified either from leaf tissue of one week old seedlings, or from dry beans that had been soaked in water for 4 hours, by the procedure of Dellaporta *et al.* [7]. The design of our PCR primers was based on the conserved amino-terminal (MASS^K/_N) and carboxy-terminal (LN^Q/_{KIL}*) amino acids of PHA-E, PHA-L,

α AI and arcelin [5]. The sequences were as follows: P1, 5'-GGCTCGAGTCTAGAGGAT^T/CT^G/TGTTG-3'; P2, 5'-CCATCGATAGATG-GCTTCCTCCAA^C/GT-3'. A 200 ng portion of each primer and 1 to 2 μ g of genomic DNA were used for PCR in the following 100 μ l reaction mixture: 60 mM Tris-HCl pH 8.5, 15 mM (NH₄)₂SO₄, 2.0 mM MgCl₂, 200 μ M each dNTP, and 2.5 units AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, CT). Conditions for PCR were 95 °C for 3 min, then 40 cycles of: 5.5 °C for 1 min, 72 °C for 1 min, and 94 °C for 1 min. The resulting PCR products were cloned directly (or specific bands were gel-purified first) in pCRII (Invitrogen, San Diego, CA), and sequenced as above.

Sequence analysis

Deduced amino acid sequences were aligned using the PILEUP and PRETTY programs of the University of Wisconsin Genetics Computer Group [8] and the dendrogram was created using the program FIGURE.

Results

Proteins that crossreact with antibodies to α AI

In seeds of the common bean, α AI is synthesized as a polypeptide of M_r 25 000 that is cotranslationally glycosylated in the endoplasmic reticulum and proteolytically processed after it arrives in the vacuoles [4]. These processing steps result in a series of polypeptides, that are either glycoforms or isoforms, in the M_r 15 000–18 000 range. Seed extracts of wild accessions of *P. vulgaris* and of other species in the genus *Phaseolus* were separated by SDS-PAGE, transferred to nitrocellulose and probed with a serum against deglycosylated red kidney bean α AI [25]. In extracts of red kidney bean (Fig. 1, lane 1) and two wild accessions (Fig. 1, lanes 2 and 3), the serum detects the polypeptides of α AI in the M_r 15 000–18 000 range, as well as possible precursors and/or ag-

gregates in the M_r 35 000 range (see [25] for discussion). The abundant protein PHA, which has about 60–65% sequence identity with α AI, is not detected by this serum (see also [35]). In wild accessions that contain arcelin (Fig. 1, lanes 4 and 5), the serum also detects polypeptides in the M_r 30 000–40 000 range, which corresponds to the position of arcelin, as well as larger polypeptides. It seems likely therefore that the serum recognizes α AI as well as arcelin polypeptides.

Tepary beans (*P. acutifolius*) contain α -amylase inhibitory activity, and seed extracts have small crossreacting polypeptides in the M_r 15 000–18 000 range (Fig. 1, lanes 6–8), as observed previously [35]. (The polypeptides in lane 8 were too faint to be observed in this image.) Extracts of the subspecies *P. acutifolius tenuifolius* contained crossreacting polypeptides in the M_r 30 000–35 000 range in addition to the M_r 15 000–18 000 polypeptides. Together these results indicate that α AI and arcelin contain common epitopes to which this particular serum contains antibodies, and that arcelin-type proteins may also be present in other bean species such as tepary bean.

Crossreacting polypeptides in the closely related species *P. polyanthus* and *P. coccineus* are shown in lanes 9 and 10 and in lanes 11 and 12, respectively. *P. polyanthus* gave a typical and strong 3-banded pattern. Both *P. coccineus* and *P. polyanthus* have α -amylase inhibitory activity (data not shown). For the five other species shown in Fig. 1, only *P. microcarpus* gave bands in the M_r 15 000–18 000 range. None of these five extracts contained inhibitory activity for porcine pancreatic or *Tenebrio molitor* α -amylase (data not shown).

Derived amino acid sequences of α AI-arcelin-PHA proteins

Two different strategies were followed to obtain derived amino acid sequences. Domesticated tepary beans and arcelin-4 type *P. vulgaris* were grown in the greenhouse and cDNA libraries were prepared using mRNA obtained from mid-maturation seeds. These libraries were screened

with an α AI (*P. vulgaris* cv. Tendergreen) probe. This resulted in the identification of the following sequences: α AI3 and arcelin-4 from arcelin-4 type *P. vulgaris*; PHA-L from *P. acutifolius*; and an arcelin-like sequence from *P. acutifolius*.

When the polymerase chain reaction was used with genomic DNA, we obtained at least two size classes of DNAs (Fig. 2) with *P. vulgaris* cv. Greensleeves: the highest-molecular-weight band represents the longer PHA sequences and the lower-molecular-weight bands represents the α AI sequence. This is consistent with the observation that these genes do not have introns and that

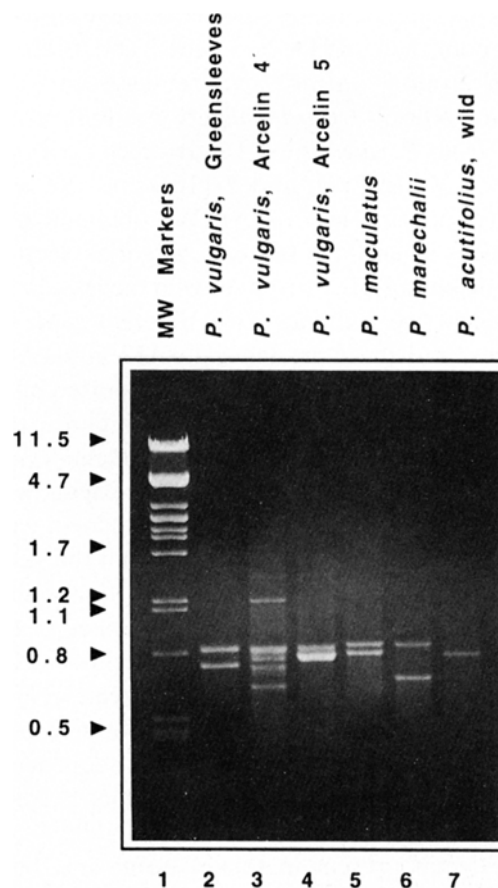


Fig. 2. PCR analysis of genomic DNA purified from various *Phaseolus* species. Ethidium bromide-stained 1.3% agarose gel showing PCR amplification products generated using primer pairs that were designed based on the conserved terminal 5' and 3' coding regions of PHA-E, PHA-L, α AI, and arcelin 1. DNA molecular weight markers are indicated in kilobase pairs on the left.

there are several short deletions in the amino acid sequence of α AI compared to that of PHA [26]. When the two bands were clearly separated for wild accessions or other species, we cloned DNA from the lower-molecular-weight band. This approach resulted in the identification of four other derived amino acid sequences: α AI-like sequences from *P. acutifolius* and *P. maculatus*; PHA-E from arcelin-5 type *P. vulgaris*; and α AI2 from arcelin-4 type *P. vulgaris*.

The derived amino acid sequences of 16 genes in the lectin-arcelin- α AI gene family are shown in Fig. 3 and the evolutionary relationships between those sequences are depicted in a dendrogram (Fig. 4). Eight of these sequences have already been published: PHA-E (dlec1) and PHA-L (dlec2) from *P. vulgaris* cv. Tendergreen [12], PHA-L (pdlec2) from *P. vulgaris* cv. Pinto [45], α AI1 (from *P. vulgaris* cv. Tendergreen [26]), arcelins 1 [30], 2 [17], and 5 [10], and the lima bean (*P. lunatus*) lectin [18]. We obtained new sequences in all three protein categories: lectins, arcelins and α AI. From a wild accession of *P. vulgaris*, we obtained two different α AI sequences, and these are labeled α AI2 and α AI3. The sequence of α AI2 was recently reported by us [24] and by Suzuki *et al.* [43]. We chose this particular accession, classified as arcelin-4, because its seeds are resistant to the Mexican bean weevil (*Zabrotus subfasciatus*), and the bean weevil (*Acanthoscelides obtectus*) [3]. From the same accession, we obtained a sequence that represents arcelin-4. The amino acid sequence of the N-terminal region of mature arcelin-4 [11] matches the derived amino acid sequence of ARL4 Pvul (w) starting from the putative processing site. On the dendrogram, this sequence is most closely related to those of arcelins 1, 2 and 5.

The lectin present in tepary beans has been purified and characterized several times [6, 34, 37]. From this species, we obtained a PHA-L related sequence (labeled PHA-L Pacu), as well as an α AI related sequence (labeled α AI-like Pacu) that is closely related to α AI of the domesticated common bean. In addition, we obtained an arcelin-like sequence from *P. acutifolius* (ARL

Pacu) indicating that the presence of arcelin sequences is not restricted to *P. vulgaris*. On the dendrogram, this sequence is more closely related to the *P. vulgaris* arcelins than to other *P. acutifolius* proteins. This finding suggests that the evolution of the arcelin sequences may not be restricted to *P. vulgaris*. We also obtained an α AI-like sequence from *P. maculatus* (α AI-like Pmac) and an α AI-like pseudo gene from *P. marechalii* (data not shown). Seed extracts of these two species do not have crossreacting polypeptides of the same size as α AI (Fig. 1B), or inhibitory activity. The *P. maculatus* sequence is more like α AI3 than α AI1 or α AI2 and the results suggest that there may be two groups of α AI proteins just as there are two groups of lectins (PHA-E and PHA-L).

Functional residues in the lectin-arcelin- α AI proteins

Four amino acids are completely conserved in all legume lectins [41] and these are shown in bold, italic type in Fig. 3. These are an aspartate residue (***d***), an asparagine residue (***N***) and two glycine residues (***G***). When any one of these residues is mutated by site-directed mutagenesis in pea lectin ([44] and J. Kijne, personal communication) or when the corresponding Asn in PHA-L is mutated to Asp, the resulting protein does not bind carbohydrate [23]. Of these four residues, the aspartate residue, essential for carbohydrate binding, is missing from all the arcelins as well as from all the α AI, indicating that these proteins are most likely not lectins. Experiments with purified arcelins show that they are very weak agglutinins [31]. It is not clear whether the weak agglutination that was observed was caused by contamination with true agglutinins in the arcelin preparation or whether the absence of the aspartate residue in arcelins creates agglutinins that are much less effective.

A unique feature of α AI is that it must be proteolytically processed to be active as an inhibitor [35]. This processing occurs in the vacuole at the asparagine residue marked by the second arrow in Fig. 3. Many legume seed proteins are processed at an asparagine residue and the process-

1	MASSNL...L	TLALFLVLIT	HANSASDTSF	NYFSNET..	..LLIQQDAT	αA12 Pval (w)	151	VqEDTF..	...srIDV	nn...ndiks	VPWDeqDYdG	200	QNAKVRITYN	αA12 Pval (w)
	MASKKcsVL	SLVFLVLIT	HANSACHTSF	NFSNET..	..LLIQQDAT	αA1-like Pacu		VEDTF..	...srIDV	nn...ndiks	VPWDeqDYdG		QNAEVRITYN	αA1-like Pacu
	MASKK...L	SLALFLVLIT	HANSALETSf	lIdafNkt..	..LLIQQDAT	αA1 Pval		VEDTF..	...srIDV	nn...ndiks	VPWDeqDYdG		QNAEVRITYN	αA1 Pval
	MASKKFTVL	..LFLVLIT	HANSANDISf	NlIdafNkt..	..LLIQQDAT	αA1-like Pmac		VafInvlyps	angRDVdVdV	nsIhpvrshq	prLRVhVipe		rqnVRITYN	αA1-like Pmac
	MASKKFTV..	..LFLVLIT	HANSATETSf	NlIdafNkt..	..LLIQQDAT	αA3 Pval (w)		VafInvlyps	angRDVdVdV	nsIhpvrshq	prLRVhVipe		rqnVRITYN	αA3 Pval (w)
	MASKK...F	tv..LFLVLIT	HANSNDIYf	NfqrFNkt..	..LLIQQDAT	PHA-L Pval (Tg)		VEDTLYMRD	WDPErHIGI	DVNSISIKT	TrWDfV...NG		ENAEVLITYD	PHA-L Pval (Tg)
	MASKN...L	SLALFLVLIT	HANSASQITf	SfdrFNkt..	..LLIQQDAT	PHA-L Pval (Hmo)		VEDTLYMRD	WDPErHIGI	DVNSISIKT	TrWDfV...NG		ENAEVLITYD	PHA-L Pval (Hmo)
	MASKN...L	SLALFLVLIT	HANSANDISf	NfqrFNkt..	..LLIQQDAT	PHA-L Pval (Hmo)		VEDTLYMRD	WDPErHIGI	DVNSISIKT	TrWDfV...NG		ENAEVLITYD	PHA-L Pval (Hmo)
	MASKN...L	SLALFLVLIT	HANSASQITf	SfdrFNkt..	..LLIQQDAT	PHA-E Pval (w)		VEDTLYMRD	WDPErHIGI	DVNSISIKT	TrWDfV...NG		ENAEVLITYD	PHA-E Pval (w)
	MASKN...L	SLALFLVLIT	HANSASQITf	SfqrFNkt..	..LLIQQDAT	PHA-E Pval (Tg)		VEDTLYMRD	WDPErHIGI	DVNSISIKT	TrWDfV...NG		ENAEVLITYD	PHA-E Pval (Tg)
	MASKN...L	SLALFLVLIT	HANSNDISf	NfqrFNkt..	..LLIQQDAT	LB12 Plun		VEDTLYMRD	WDPErHIGI	DVNSISIKT	TrWDfV...NG		ENAEVLITYD	LB12 Plun
	MASKN...L	SLALFLVLIT	HANSNDISf	NfqrFNkt..	..LLIQQDAT	AR1 Pval (w)		VEDTLYMRD	WDPErHIGI	DVNSISIKT	TrWDfV...NG		ENAEVLITYD	AR1 Pval (w)
	MASKK...L	SLALFLVLIT	HANSATETSf	NfqrFNkt..	..LLIQQDAT	AR2 Pval (w)		VEDTLYMRD	WDPErHIGI	DVNSISIKT	TrWDfV...NG		ENAEVLITYD	AR2 Pval (w)
	MASKK...L	SLALFLVLIT	HANSATETSf	NfqrFNkt..	..LLIQQDAT	AR5 Pval (w)		VEDTLYMRD	WDPErHIGI	DVNSISIKT	TrWDfV...NG		ENAEVLITYD	AR5 Pval (w)
	MASKK...L	SLALFLVLIT	HANSATETSf	NfqrFNkt..	..LLIQQDAT	AR4 Pval (w)		VEDTLYMRD	WDPErHIGI	DVNSISIKT	TrWDfV...NG		ENAEVLITYD	AR4 Pval (w)
	MASKK...L	SLALFLVLIT	HA...catf	dPpTfHkcd	..LLIQQDAT	ARL Pacu		VEDTLYMRD	WDPErHIGI	DVNSISIKT	TrWDfV...NG		ENAEVLITYD	ARL Pacu
	M-SS-----	---L-L--L-	-----F	---F	---L-L-Q--A-	Consensus		VEDTLYMRD	WDPErHIGI	DVNSISIKT	TrWDfV...NG		ENAEVLITYD	Consensus
51	VSSKGVLqL	hTV	DSMcAFYSA	PIQIRDSTG	NVAsPDtNFT	αA12 Pval (w)	201	SSTKvLAVSL	snPSTGKSne	V..SArMvEVEK	eLDDWvrvGF	250	SAISGvheys	αA12 Pval (w)
	VSSNgLnLqL	hTm	DSMcAFYSA	PIQIRDSTG	NVAsPDtNFT	αA1-like Pacu		SSTKvLAVSL	snPSTGKSne	V..SArMvEVEK	eLDDWvrvGF		ignSgVhays	αA1-like Pacu
	VSSNgLnLqL	hTm	DSMcAFYSA	PIQIRDSTG	NVAsPDtNFT	αA1 Pval		SSTKvLAVSL	snPSTGKSne	V..SArMvEVEK	eLDDWvrvGF		SATSGayqws	αA1 Pval
	VSSNgLnLqL	hTm	DSMcAFYSA	PIQIRDSTG	NVAsPDtNFT	αA1-like Pmac		SSTKvLAVSL	snPSTGKSne	V..SArMvEVEK	eLDDWvrvGF		SATSGayqws	αA1-like Pmac
	VSSNgLnLqL	hTm	DSMcAFYSA	PIQIRDSTG	NVAsPDtNFT	αA3 Pval (w)		SSTKvLAVSL	snPSTGKSne	V..SArMvEVEK	eLDDWvrvGF		SATSGayqws	αA3 Pval (w)
	VSSNgLnLqL	hTm	DSMcAFYSA	PIQIRDSTG	NVAsPDtNFT	PHA-L Pval (Tg)		SSTKvLAVSL	snPSTGKSne	V..SArMvEVEK	eLDDWvrvGF		SATSGayqws	PHA-L Pval (Tg)
	VSSNgLnLqL	hTm	DSMcAFYSA	PIQIRDSTG	NVAsPDtNFT	PHA-L Pval (Hmo)		SSTKvLAVSL	snPSTGKSne	V..SArMvEVEK	eLDDWvrvGF		SATSGayqws	PHA-L Pval (Hmo)
	VSSNgLnLqL	hTm	DSMcAFYSA	PIQIRDSTG	NVAsPDtNFT	PHA-L Pval		SSTKvLAVSL	snPSTGKSne	V..SArMvEVEK	eLDDWvrvGF		SATSGayqws	PHA-L Pval
	VSSNgLnLqL	hTm	DSMcAFYSA	PIQIRDSTG	NVAsPDtNFT	PHA-E Pval (w)		SSTKvLAVSL	snPSTGKSne	V..SArMvEVEK	eLDDWvrvGF		SATSGayqws	PHA-E Pval (w)
	VSSNgLnLqL	hTm	DSMcAFYSA	PIQIRDSTG	NVAsPDtNFT	PHA-E Pval (Tg)		SSTKvLAVSL	snPSTGKSne	V..SArMvEVEK	eLDDWvrvGF		SATSGayqws	PHA-E Pval (Tg)
	VSSNgLnLqL	hTm	DSMcAFYSA	PIQIRDSTG	NVAsPDtNFT	LB12 Plun		SSTKvLAVSL	snPSTGKSne	V..SArMvEVEK	eLDDWvrvGF		SATSGayqws	LB12 Plun
	VSSNgLnLqL	hTm	DSMcAFYSA	PIQIRDSTG	NVAsPDtNFT	AR1 Pval (w)		SSTKvLAVSL	snPSTGKSne	V..SArMvEVEK	eLDDWvrvGF		SATSGayqws	AR1 Pval (w)
	VSSNgLnLqL	hTm	DSMcAFYSA	PIQIRDSTG	NVAsPDtNFT	AR2 Pval (w)		SSTKvLAVSL	snPSTGKSne	V..SArMvEVEK	eLDDWvrvGF		SATSGayqws	AR2 Pval (w)
	VSSNgLnLqL	hTm	DSMcAFYSA	PIQIRDSTG	NVAsPDtNFT	AR5 Pval (w)		SSTKvLAVSL	snPSTGKSne	V..SArMvEVEK	eLDDWvrvGF		SATSGayqws	AR5 Pval (w)
	VSSNgLnLqL	hTm	DSMcAFYSA	PIQIRDSTG	NVAsPDtNFT	AR4 Pval (w)		SSTKvLAVSL	snPSTGKSne	V..SArMvEVEK	eLDDWvrvGF		SATSGayqws	AR4 Pval (w)
	VSSNgLnLqL	hTm	DSMcAFYSA	PIQIRDSTG	NVAsPDtNFT	ARL Pacu		SSTKvLAVSL	snPSTGKSne	V..SArMvEVEK	eLDDWvrvGF		SATSGayqws	ARL Pacu
	VSSNgLnLqL	hTm	DSMcAFYSA	PIQIRDSTG	NVAsPDtNFT	Consensus		SSTKvLAVSL	snPSTGKSne	V..SArMvEVEK	eLDDWvrvGF		SATSGayqws	Consensus
101	NSvIGLdFAL	VPV...QPKsK	GB.....	TVT	150	αA12 Pval (w)	251	fETRDVLSWS	FSSKFS...qh	TT...SERSNI	LLNQIL*	287	αA12 Pval (w)	
	NSvIGLdFAL	VPV...QPKsK	GB.....	TVT	150	αA1-like Pacu		fETRDVLSWS	FSSKFS...qh	TT...SERSNI	LLNQIL*		αA1-like Pacu	
	NSvIGLdFAL	VPV...QPKsK	GB.....	TVT	150	αA1 Pval		fETRDVLSWS	FSSKFS...qh	TT...SERSNI	LLNQIL*		αA1 Pval	
	NSvIGLdFAL	VPV...QPKsK	GB.....	TVT	150	αA1-like Pmac		fETRDVLSWS	FSSKFS...qh	TT...SERSNI	LLNQIL*		αA1-like Pmac	
	NSvIGLdFAL	VPV...QPKsK	GB.....	TVT	150	αA3 Pval (w)		fETRDVLSWS	FSSKFS...qh	TT...SERSNI	LLNQIL*		αA3 Pval (w)	
	NSvIGLdFAL	VPV...QPKsK	GB.....	TVT	150	PHA-L Pval (Tg)		fETRDVLSWS	FSSKFS...qh	TT...SERSNI	LLNQIL*		PHA-L Pval (Tg)	
	NSvIGLdFAL	VPV...QPKsK	GB.....	TVT	150	PHA-L Pval (Hmo)		fETRDVLSWS	FSSKFS...qh	TT...SERSNI	LLNQIL*		PHA-L Pval (Hmo)	
	NSvIGLdFAL	VPV...QPKsK	GB.....	TVT	150	PHA-L Pval		fETRDVLSWS	FSSKFS...qh	TT...SERSNI	LLNQIL*		PHA-L Pval	
	NSvIGLdFAL	VPV...QPKsK	GB.....	TVT	150	PHA-E Pval (w)		fETRDVLSWS	FSSKFS...qh	TT...SERSNI	LLNQIL*		PHA-E Pval (w)	
	NSvIGLdFAL	VPV...QPKsK	GB.....	TVT	150	PHA-E Pval (Tg)		fETRDVLSWS	FSSKFS...qh	TT...SERSNI	LLNQIL*		PHA-E Pval (Tg)	
	NSvIGLdFAL	VPV...QPKsK	GB.....	TVT	150	LB12 Plun		fETRDVLSWS	FSSKFS...qh	TT...SERSNI	LLNQIL*		LB12 Plun	
	NSvIGLdFAL	VPV...QPKsK	GB.....	TVT	150	AR1 Pval (w)		fETRDVLSWS	FSSKFS...qh	TT...SERSNI	LLNQIL*		AR1 Pval (w)	
	NSvIGLdFAL	VPV...QPKsK	GB.....	TVT	150	AR2 Pval (w)		fETRDVLSWS	FSSKFS...qh	TT...SERSNI	LLNQIL*		AR2 Pval (w)	
	NSvIGLdFAL	VPV...QPKsK	GB.....	TVT	150	AR5 Pval (w)		fETRDVLSWS	FSSKFS...qh	TT...SERSNI	LLNQIL*		AR5 Pval (w)	
	NSvIGLdFAL	VPV...QPKsK	GB.....	TVT	150	AR4 Pval (w)		fETRDVLSWS	FSSKFS...qh	TT...SERSNI	LLNQIL*		AR4 Pval (w)	
	NSvIGLdFAL	VPV...QPKsK	GB.....	TVT	150	ARL Pacu		fETRDVLSWS	FSSKFS...qh	TT...SERSNI	LLNQIL*		ARL Pacu	
	NSvIGLdFAL	VPV...QPKsK	GB.....	TVT	150	Consensus		fETRDVLSWS	FSSKFS...qh	TT...SERSNI	LLNQIL*		Consensus	

Fig. 3. Multiple alignment of deduced amino acid sequences of the 16 genes in the *Phaseolus* lectin-arcelin-αA1 gene family. The alignment was generated using UWGCG's PILEUP and PRETTY programs. Eight of these sequences have already been published; the names of the new sequences are indicated in bold. The four amino acids that are essential for carbohydrate binding and are completely conserved in all legume lectins are shown in bold italics. The arrows represent sites of processing for removal of the signal peptides and the processing site utilized to yield active αA1. Pval, *P. vulgaris*; Pacu, *P. acutifolius*; Pmac, *P. maculatus*; Plun, *P. lunatus*; w, wild; TG, Tendergreen.

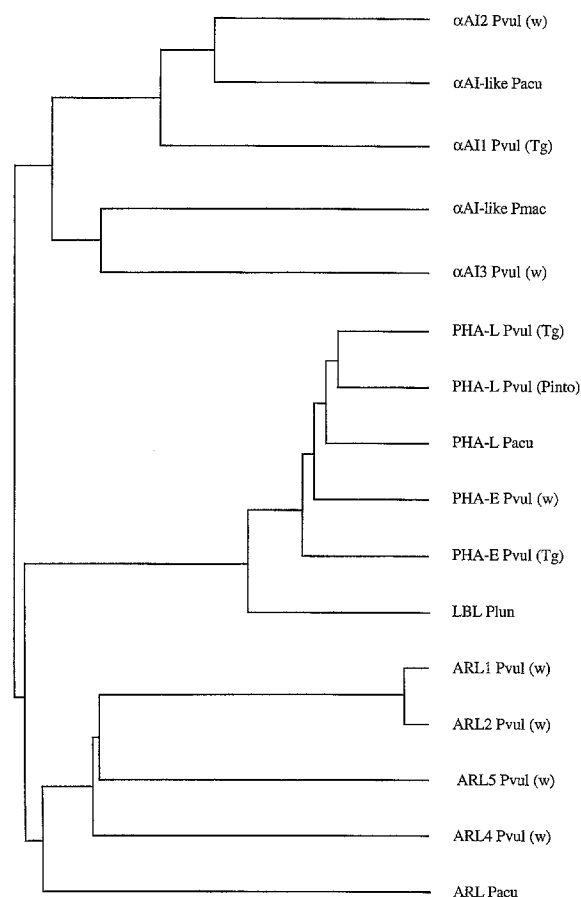


Fig. 4. Cluster dendrogram by UWGCG's PILEUP and FIGURE programs, based on the amino acid sequence alignment from Fig. 3. The program aligns the sequences using progressive, pairwise alignments using the method of Needleman and Wunsch [28]. The dendrogram is generated from these alignments and shows the clustering using the clustering strategy Unweighted Pair Group Method using Arithmetic Averages. Abbreviations are as in Fig. 3.

ing protease has recently been purified [27, 39]. This asparagine residue is present in all the α AI proteins that are grouped together in Fig. 4, as well as in arcelins 1 and 2 but it is absent from the lectins as well as from arcelins 4 and 5. In all the proteins mentioned, asparagine is followed by serine and processing occurs between these two residues. Arcelins are not proteolytically processed and the results therefore indicate that the Asp-Ser motif in this position is not by itself sufficient to ensure such processing. This may account for the observation that *P. maculatus* and

P. marechalii do not contain polypeptides in the M_r 15 000–18 000 range although they contain sequences with the asparagine-serine motif in the correct position. The alternative explanation is that these sequences are not expressed at a sufficiently high level to detect the proteins with the methods employed here, or that these particular plant species lack the processing protease.

Gaps in the aligned sequence

The α AI protein has three gaps compared to the lectins and these are labeled as Gap 1, Gap 2 and Gap 3 in Fig. 4. Gap 1 appears to be unique to all the proteins grouped together as α AIs. The second gap, Gap 2, found in α AI1, is also found in α AI2 (α AI from wild accession of *P. vulgaris*) but is absent from α AI3 and the α AI-like sequences of the other species. The third gap, Gap 3, is not unique to α AI but is also found in all the arcelins, as well as in the arcelin-like protein of *P. acutifolius*. This gap is found in the α AI2 and α AI-like protein of *P. acutifolius*, but not in α AI3 or the α AI-like protein of *P. maculatus*. The presence or absence of these gaps accounts to a large extent for the grouping of the α AI sequences in two groups (Fig. 4).

Discussion

Many seeds contain plant defense proteins, and the PHA-arcelin- α AI family of plant defense proteins was first identified in the common bean *P. vulgaris* [26]. Lectins such as PHA are known to occur in many species of legumes and are probably present in all species of the genus *Phaseolus*. They have been studied in some detail only in *P. vulgaris*, *P. lunatus* (lima bean) and in *P. acutifolius*. The three members of this gene family occur at a single locus in the *P. vulgaris* genome, suggesting that the genes may have arisen by duplication [29]. It would be of considerable interest to understand how this gene family has evolved. This may be a highly evolved protein family with multiple members in each species. Some mem-

bers of this family may encode agglutinins and other members inhibitors of α -amylase. A comparison of the new sequences we have obtained with the already known sequences allows us to draw several conclusions.

First, there do seem to be three groups of sequences, although these groupings may disappear when we obtain more sequences from other wild accessions and especially from other species. Indeed, a cDNA initially identified as encoding lima bean lectin [15] but apparently not encoding the true lima bean lectin [18] falls outside these three groups (data not shown). On the other hand, the PHA sequence from tepary bean (PHA-L Pacu) and the real lima bean lectin (LBL) [18] are more closely related to PHA of the common bean than to α AI or arcclin proteins. Since the *P. acutifolius* PHA sequence has the four conserved amino acids found in all lectins, it is likely to encode an agglutinin.

Second, two different α AI sequences were found in the wild accession of *P. vulgaris* G12949. This arcclin-4 type bean is resistant to the bean weevil (*A. obtectus*) and the Mexican bean weevil (*Z. subfasciatus*) and it is possible that this resistance to weevil infestation results from specific α AI. Indeed, arcclin-type beans contain variants of α AI that inhibit the α -amylases of these weevils [42]. Our findings indicate that the bean genome harbors more than one α AI gene, just as it has more than one lectin gene. This confirms biochemical work that many cultivars contain two distinct forms of the inhibitor [14]. Amino acid sequencing of α AI purified from seed meal of *P. vulgaris* cv. Rico 23 shows the presence of two different proteins, each with its own α and β subunits (F. Finardi-Filho, unpublished results). Whether α AI2, α AI3 and other α AI-like sequences actually encode inhibitors can only be demonstrated by expressing these genes in a biological system that allows for the necessary post-translational modifications and activation of α AI (e.g. tobacco seeds), as was done for α AI1 [1]. However, the identification of the biological source of the α -amylase that is inhibited by each α AI may not be a trivial matter. For example, we found that α AI2 (expressed in tobacco seeds,

under the control of the PHA-L promoter) does not inhibit the α -amylases of *Tenebrio molitor*, *A. obtectus*, *Z. subfasciatus*, or the α -amylase found in porcine pancreas [24].

Third, our results show that arcclin-4 is more closely related to the recently reported sequence of arcclin-5 than to the arcclin-1 and -2 proteins [10]. Indeed, the bean arcclins appear to be a less closely related group of proteins than the bean phytohemagglutinins sequenced so far. Arcclin proteins may not be restricted to wild accessions of the common bean; seed extracts of the tepary bean have proteins in the M_r 30 000–35 000 range that crossreact with the α AI serum just like the wild accessions of *P. vulgaris* that contain arcclin. Additionally, an arcclin-like sequence was obtained from *P. acutifolius*. We therefore suggest that arcclin-like sequences may also be present in domesticated varieties of the common bean, but that these may be poorly expressed in the seeds. Thus, the main difference between arcclin-type accessions and other accessions of *P. vulgaris* may be the copy number of the arcclin genes and the promoter strength.

The total number of lectin and lectin-like genes in the *P. vulgaris* genome is not known. Gene mapping indicates that PHA, α AI and arcclin occur at the same locus, although lectin genes may also occur elsewhere in the genome [29]. The two phytohemagglutinin genes that encode PHA-E and PHA-L are known to be linked and arose most likely from a recent duplication [12]. Genomic cloning revealed the presence of three lectin-like genes on a single 19 kb fragment of *P. vulgaris* cv. Pinto DNA [45]. Analysis of the entire locus and of the lectin-like genes elsewhere in the genome is needed to understand how these genes may have evolved.

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