Molecular and functional analysis of α-amylase inhibitor genes and proteins in the common bean *Phaseolus vulgaris*

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**ABSTRACT**  
Bean (*Phaseolus vulgaris*) seeds accumulate three evolutionarily related plant defense proteins: phytohemaglutinin (PHA), arcelin (Arc) and α-amylase inhibitor (αAI). We identified the gene that encodes snap bean (cv. Greensleeves) αAI on the basis of a partial amino acid sequence of purified αAI supplied by John Whitaker (UC Davis), and the expression of the gene in tobacco seeds. Tobacco seeds expressing this gene contained αAI activity, whereas control tobacco seeds do not. These findings allowed us to conclude that αAI is a proteolytically processed glycoprotein that has as its precursor a 27 kD polypeptide. The amino acid sequence shows 61% identity with Arc and 52% identity with PHA. Proteolytic processing of αAI occurs in the vacuole and is necessary for the activation of αAI. We have now cloned a new cDNA from an arcelin-type accession (G-12949) that has 77% sequence identity to the Greensleeves αAI and probably encodes a novel αAI. When this gene is expressed in tobacco seeds, the protein does not inhibit porcine or *Tenebrio molitor* α-amylase. Experiments conducted in collaboration with L. Murdock and R. Shade (Purdue University) show that the αAI from Greensleeves is a potent inhibitor of the development of *Callosobruchus maculatus* larvae in artificial seeds based on cowpea meal. Levels of 0.7% are sufficient to arrest the development of the larvae. In collaboration with H. Schroeder and T.J. Higgins (CSIRO, Canberra, Australia) we have made transgenic pea (*Pisum sativum*) plants that express the Greensleeves αAI in their seeds. Analysis by R. Shade and L. Murdock shows that many of the seeds from the M2 generation are resistant against the larvae of the cowpea and the azuki bean weevils. The degree of resistance correlates with the level of αAI in the transgenic seeds. The potential for using new αAI genes from wild accessions to make legume crops resistant to various bruchid species will be discussed.

Bruchids are the most serious pests attacking food legume seeds during prolonged storage under less than ideal conditions. In the Americas, the Mexican bean weevil (*Zabrotes subfasciatus*), and the Bean weevil (*Acanthoscelides obtectus*) do considerable damage to the domesticated common bean, *Phaseolus vulgaris*, because it lacks the necessary antibiosis.
factors. Such antibiotics factors are found in wild accessions of the common bean (Dobie et al., 1990; Schoonhoven et al., 1983). In addition, the domesticated varieties contain antibiotics factors against Old World bruchids such as the cowpea weevil (Callosobruchus maculatus) and the azuki bean weevil (C. chinensis). These weevils cause considerable damage to the Old World legumes such as the cowpea, the chickpea and the mung bean. Our laboratory has been involved in identifying proteinaceous antibiotics factors in Phaseolus vulgaris with the ultimate aim of transferring their genes either by classical plant breeding methods or via genetic engineering.

Seeds generally contain a variety of antibiotics factors because plants have evolved a multiplicity of defenses against their pests and pathogens. The seeds of the common bean contain several related proteins that function in the defense of the plant against predators and insects. These proteins are the phytohemagglutinins PHA-E and PHA-L, arcelin (Arc) and α-amylase inhibitor (αAI). The amino acid sequences of these proteins show a high degree of sequence identity, suggesting that the proteins are evolutionarily related. As the genes for these proteins are also linked in the genome, it is reasonable to assume that they arose by gene duplication and then evolved and acquired their distinctive biological functions. PHA-E and PHA-L are lectins that bind to the complex glycans found on plasma membrane glycoproteins of many mammalian cells. PHA-L agglutinates white blood cells and PHA-E agglutinates red blood cells. These lectins are toxic when fed to mammals and birds. This toxicity is presumed to be the result of their lectin activity. In other words, it is their binding to the microvilli of the intestinal tract of mammals that sets in motion a sequence of events that results in their toxicity.

The biological activity of αAI is very different from that of PHA even though the two proteins are homologous. It forms a 1:1 complex with certain α-amylases. This binding is very tight (K_d = 3.1 ¥ 10^{-5} M) and has a pH optimum around 5.0 (Lajolo and Finardi Filho, 1985; Wilcox and Whitaker, 1984). The bean αAI inhibits α-amylases of mammals and of certain insects. The α-amylases of other insects and those of plants are not inhibited. In artificial seeds made out of cowpea flour, relatively low levels of αAI (0.5 %) prevent the development of bruchid larvae of Callosobruchus maculatus, suggesting that αAI is responsible for the resistance of the common bean to this species of bruchid (Ishimoto and Kitamura, 1989; Huesing et al., 1991), rather than PHA as had been previously reported (Janzen et al., 1976). It appears that preparations of PHA used in earlier experiments were contaminated with αAI.
Experiments by Hartweck et al., (1991) show that purified arcelins are weak agglutinins in comparisons to PHA. The agglutination obtained in these experiments could have been caused by a low level contamination of the purified arcelin with PHA. Using artificial seeds, Osborn et al., 1988, showed that arcelin at a level of 10 % retards the development of bruchid larvae, and he postulated that arcelin may be the toxic principle in wild accessions of the common bean. The various wild accessions that have been collected have six different forms of arcelin as shown by the electrophoretic mobility of the polypeptides; these different bean genotypes also have different levels of antibiosis towards bruchid larvae. For example, in one study Arc5 had the highest level of antibiosis resistance to Zabrotes subfaciatus, followed by Arc4, Arc1 and Arc2. Arc3 genotypes had the lowest level of resistance (Cardona et al., 1990). It is likely that these beans contain more than one arc gene product as well as more than one αAl gene product. The various proteins are likely to differ in their biological specificity, making the task of identifying the toxicity of each protein towards a specific insect species particularly difficult (Figure 1).

Methodology

In our research we have taken a different approach to determining the biological activities of the proteins in this family. We have begun to clone their genes, express them in the seeds of another plant species such as tobacco, oilseed rape or pea, and then determine the biological activity of the protein in this neutral background. Here we report the isolation of a new gene in the bean lectin family from an arcelin 4 genotype and its expression in tobacco seeds. The protein has extensive amino acid homology with the αAl found in the domestic bean but, does not inhibit the activity of α-amylase from the porcine pancreas or from Zabrotes subfasciatus. Nevertheless, sequence comparisons identify the protein encoded by this gene as likely to be an αAl. We have also isolated a new gene from an arcelin 5 genotype which is highly homologous to PHA-E found in the domesticated bean.
Figure 1. Amino acid sequence comparisons. (A) αAl-2 from the arcelin4 genotype is on top and αAl from the domesticated cultivar (Greensleeves) is on the bottom. (B) PHA-E from the arcelin 5 genotype is on top and PHA-E from Greensleeves is on the bottom.
Results and discussion

Isolation and Characterization of Lectin-like Genes

A PCR based approach, utilizing genomic DNA from arcelin 4 and 5 genotypes, was used to clone new genes in the PHA family. PCR primers were based on the conserved amino-terminal and carboxy-terminal amino acids of PHA-E, PHA-L, \( \alpha \)AI, and Arc 1 (Chrispeels and Raikhel, 1991). The resulting clones were screened by partial sequence analysis, and based on this screening, 2 clones (one from the Arc 4 genotype G12949 and one from the Arc 5 genotype G2771) were selected for further characterization. The clone isolated from the Arc 4 genotype has 85% amino acid sequence similarity with the \( \alpha \)AI from the domestic bean (Figure 1A) and will be referred to as \( \alpha \)AI-2, while the clone isolated from the Arc 5 genotype has 90% amino acid sequence similarity with PHA-E from the domestic bean (Figure 1B). This PHA-E clone has not been further characterized.

As these were genomic clones rather than cDNA clones obtained from RNA in developing beans, we needed to determine if the \( \alpha \)AI-2 gene is actually expressed in arcelin 4 beans. To demonstrate the expression of \( \alpha \)AI-2 in developing arcelin 4 beans, we utilized RNase protection. The results of this analysis are shown in Figure 2, and it is clear that the \( \alpha \)AI-2 gene is expressed at high levels in arcelin 4 beans.

Figure 2. RNase protection assay with \( \alpha \)AI-2. The \(^{32}\)P]UTP probe is in vitro synthesized (–) sense RNA from the \( \alpha \)AI-2 clone containing 315 nt of sequence complementary to the \( \alpha \)AI-2 gene. This probe was then hybridized as follows: Lane 1 = with 10 \( \mu \)g yeast tRNA, incubated with RNase digestion buffer only; Lane 2 = with 10 \( \mu \)g yeast tRNA, incubated with RNase; Lane 3 = with 0.5 \( \mu \)g in vitro synthesized \( \alpha \)AI-2 sense RNA, digested with RNase; Lane 4 = with 6 \( \mu \)g total RNA from developing Arc 4 beans, digested with RNase; and Lane 5 = with 12 \( \mu \)g total RNA from developing Arc 4 beans, digested with RNase.
Figure 3. Immunoblot analysis with antibodies against αAl. Lane 1 = extract from untransformed tobacco seeds; Lane 2 = extract from tobacco seeds transformed with αAl-2 gene (from G12949 P. vulgaris); and Lane 3 = extract from G12949 P. vulgaris seeds.

Biological activity of a putative αAl protein.

To determine the biological activity of the protein encoded by the αAl-2 gene, we expressed the gene in tobacco seeds. This was accomplished by fusing the promoter of PHA-L to the coding region of the gene, moving this construct into an appropriate plant transformation vector, and then using Agrobacterium mediated transformation to obtain transgenic tobacco plants that express αAl-2 in their seeds. Immunoblot analysis using antisera raised against αAl from the domestic bean (Figure 3) showed that αAl-2 accumulated to high levels in the seeds of these transgenic tobacco plants. The polypeptide pattern of the protein found in tobacco is somewhat different from that found in beans (compare the lower 3 bands in lane 3 with the bands in lane 2). This difference may be caused by the presence of more than one αAl in the seeds, or to a difference in the proteolytic processing of the protein. The former seems likely as we recently obtained a second αAl cDNA from an arcelin4 seed cDNA library. This gene is also highly homologous to αAl from domestic beans, and is referred to as αAl-3.
Table 1. α-Amylase inhibitory activity of bean seed and transgenic tobacco seed extracts.

<table>
<thead>
<tr>
<th>Source of Extract</th>
<th>Porcine Pancreas</th>
<th>Source of Enzyme</th>
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<tr>
<td></td>
<td></td>
<td>Tenebrio Acanthoscelides molitor</td>
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<tr>
<td>Domesticated <em>P. vulgaris</em> (Ica Pijao) seeds</td>
<td>+</td>
<td>Zabrotes obtectus</td>
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<tr>
<td>Tobacco seeds transformed with α-Al gene from domesticated <em>P. vulgaris</em></td>
<td>+</td>
<td>subfasciatus</td>
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<tr>
<td>G12949 Arc4 <em>P. vulgaris</em> seeds</td>
<td>+</td>
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<tr>
<td>Tobacco seeds transformed with α-Al gene from G12949 Arc4 <em>P. vulgaris</em></td>
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Proteins extracted from tobacco and bean seeds in a buffer containing 10 mM β-mercaptoethanol.

To test the biological activity of the αAl we prepared protein extracts from the tobacco seeds and assayed their αAl activity with porcine pancreas α-amylase and with the α-amylases from *Tenebrio molitor, A. obtectus* and *Z. subfasciatus*. The results, summarized in Table I indicate that αAl-2 lacks any inhibitory activity with the enzymes tested, in contrast to tobacco seed extracts obtained from plants transformed with the αAl gene from domesticated beans. This negative result does not mean that the protein in not an amylase inhibitor, although that is a distinct possibility. It may be that we have not identified the correct source of α-amylase or that this particular gene is an evolutionary intermediate that has not yet acquired inhibitory activity. The evolutionary relationship between the two new genes (αAl-2 and PHA-E) of these wild accessions, and the already known phytohemagglutinin, arcelin and aAl genes from domesticated cultivars is shown in Figure 4.

Our future work will be directed at isolating additional αAl genes from the Arc4 genotypes and testing the biological specificity of the protein. We are also attempting to define the active site of αAl. This protein has the interesting property that activation of its inhibitory activity requires proteolytic processing (Puyecto *et al.*, 1993). We postulate that this processing step brings together specific residues in both subunits to create the active site.
Figure 4. Evolutionary relationship between the different arcelin, αAI and PHA genes of *P. vulgaris* and lima bean lectin.

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References


