Do Legume Storage Proteins Play a Role in Defending Seeds against Bruchids?[1]

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The seeds of plants are rich stores of proteins, carbohydrates, and lipids and are therefore used by heterotrophs as valuable food sources. Humans use seeds as a major food source and have learned, through agricultural practice, how to increase the levels and the quality of their components. They have also learned how to deal with the multiplicity of toxic or antinutritional compounds present in seeds. It is believed that these seeds, most of which are not essential for the establishment of the new plant following germination, contribute to the protection and defense of seeds against pathogens and predators. However, insects, fungi, and bacteria have also learned how to cope with detrimental compounds in order to take advantage of the high nutritional value of seeds.

Coleopteran insects of the family Bruchidae, the seed weevils, have been associated with the seeds of leguminous plants through co-evolutionary processes. These processes have permitted the weevils to thrive on seeds full of toxic compounds, in contrast to the majority of the other potential aggressors, which are incapable of dealing with them. The association between bruchids and legume seeds is highly specific with only seeds of a very few species being attacked by any one insect species.

Among our food sources, plants of the legume family contribute some of the most important protein-rich seeds. The common bean (*Phaseolus vulgaris*), native of the New World and the cowpea (*Vigna unguiculata*), which originated in Africa, are heavily attacked by bruchids, both in the field and in storage. Infestations are commonly so heavy that the seeds are unsuitable for use as food, feed, or planting.

Control of bruchid infestation is done by treating stored seeds with methyl bromide, carbon disulfide, and several other chemicals. These are considered environmentally undesirable and are too expensive for subsistence farmers. To increase the insect resistance of cultivated varieties plant breeders are interested in understanding resistance mechanisms that operate in wild varieties or why certain bruchids attack one cultivated species but not another.

Both the common bean and cowpea are endowed with compounds called general defensive compounds that protect their seeds against widely different herbivores. Among these are the tannins, cyanogenic glucosides, non-protein amino acids, and proteins such as protease and amylase inhibitors, lectins, chitinases, β-1,3-glucanases. These defensive compounds are ineffective against the host-specific bruchids, *Callosobruchus maculatus* and *Zabrotes subfasciatus*, which attack cowpea and common bean, respectively. Host-specific defenses are rare and are generally found in populations in the centers of dispersion of the particular plant species. Landraces of cowpea and common bean that produce seeds resistant to their associated bruchids have been discovered respectively in West Africa and Mexico. The biochemical basis of the resistance of cowpea and common bean seeds to *C. maculatus* and *Z. subfasciatus*, respectively, is the focus of this *Update*.

DIFFERENT TYPES OF PROTEINS MAKE UP THE RESERVES OF SEEDS

Seeds contain proteins of several kinds that are laid down during development. Among these are the storage proteins, which are traditionally thought of as being metabolically inactive. Storage proteins are mostly the classically known globulins, which are insoluble in water and are typically present in leguminous seeds. Prolamins and glutelins, which are alcohol and alkali soluble, respectively, are found mostly in cereal seeds. Many water-soluble albumins have also been classified as reserve proteins. Other kinds of seed proteins, which are also used for their nitrogen and carbon, are associated with defense

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mechanisms that plants have developed against the action of pests and pathogens. They are proteinase inhibitors, lectins, lectin-like proteins (arcelins [Arc] and α-amylase inhibitors [αAI]), ribosome-inactivating proteins, allergens, lipid transfer proteins, glucanases, and chitinases. Seed proteins also include hydrolases like amylases, proteinases, and lipases that act in the mobilization of the several types of associated reserve compounds, the products of which are used during germination for the synthesis of new tissues (Shewry et al., 1995).

VICILINS, LEGUMINS, AND ARCS, THE ABUNDANT TYPICAL STORAGE PROTEINS OF LEGUME SEEDS

Legume seeds characteristically contain storage proteins of the legumin and vicilin types. These are salt-soluble globulins of which the legumins are formed by two chains, linked by disulfide bonds. They are generally encoded by a large number of genes whose translation products are proteolytically processed to the final two-chain proteins. Legumins aggregate to form hexamers and are also known as 11S storage globulins (Shewry et al., 1995).

Vicilins, also known as 7S storage globulins, are usually single-chain proteins without disulfide bonds. They aggregate to form trimers of subunits with varying molecular masses (45–53 kD). They are also encoded by a large number of genes. The derived amino acid sequences of vicilin subunits from leguminous and other plant species show a high degree of sequence identity indicating their evolutionary relatedness (Shewry et al., 1995). The N-terminal portions of vicilin sequences show similarity to the C-terminal regions suggesting that the vicilins arose from a gene duplication event (Shewry et al., 1995; Shutov et al., 1995). Three-dimensional structures of several of these proteins have been solved by x-ray crystallography confirming the symmetry of the molecule and showing the arrangement of the subunits in the trimer (Ng et al., 1993).

There is also sequence identity between legumins and vicilins, both at the amino acid and nucleotide levels. Therefore it seems that legumins and vicilins share a common ancestor (Shewry et al., 1995; Shutov et al., 1995). It is also thought that other functionally non-related proteins, such as spherulin-like proteins of myxomycetes, which are involved in cellular desiccation processes, germins, and Suc-binding proteins originated from this same ancestor since they all exhibit sequence similarity to the legumins and vicilins from seeds (Shutov et al., 1995; Braun et al., 1996).

The major seed storage proteins of common bean are phaseolin (vicilin-like 7S globulin) and phytohemagglutinins (PHA). Some wild accessions from Mexico contain a third abundant protein, Arc, and its presence at high concentrations seems to be related to the low amount of phaseolin in the seeds. In these seeds, Arc replaces phaseolin as the storage protein. Arc, PHA, and αAI comprise the bean-lectin family. Despite some common features, these related proteins differ in their mode of action. PHA is a lectin that binds to glycans of the intestinal mucosa of mammals and act as mitogen; αAI inhibits the activity of certain mammalian and insect α-amylases, whereas Arc was suggested to bind to the peritrophic matrix of the gut of insects and interfere with nutrient absorption (Higgins et al., 1998). Since αAIs exhibit no sugar-binding sites and Arcs show weak agglutinin properties, they are called lectin-like proteins (Hamelryck et al., 1996).

Although the genes that encode these proteins are tightly linked at one locus in linkage group B4, other members of this gene family are dispersed in the bean genome since two other members are mapped on linkage group B7. It is likely that these homologous genes have arisen by duplication of an ancestral gene but it is not known when they diverged (Freyre et al., 1998).

VICILINS FROM SOME COWPEA AND SEVERAL OTHER LEGUMES IMPAIR DEVELOPMENT OF *CALLOSOBRUCHUS MACULATUS*

The observation that legume vicilins bind to a chitin matrix led to the discovery that vicilins, from cowpea and other legumes, strongly bind to several chitin-containing structures. Among these are structures of the midgut of both *C. maculatus* and *Z. subfasciatus* (Firmino et al., 1996) and cell walls or plasma membranes of filamentous fungi and yeasts (Gomes et al., 1998). Vicilins isolated from *C. maculatus*-resistant cowpea seeds and from several other legumes (Yunes et al., 1998) slow down larval development of this insect. Binding of vicilins to chitin-containing structures is prevented by sugars such as Glc, GlcNAc, and Suc, suggesting that competition for the same sugar-binding sites occurs (Miranda et al., 1998). We also found that chemical modification of Trp residues of bruchid-resistant vicilins by iodine or *N*-bromosuccinimide strongly reduces binding to chitin matrices and reduces the deleterious effects on insect development indicating that this amino acid is present at the binding site(s) for chitin in vicilins (Miranda et al., 1998).

The involvement of vicilins in the resistance of cowpea (TVu 2027 line) seeds to *C. maculatus* was first suggested during investigations into the possible roles of trypsin inhibitors in this process. We found no correlation between the levels of these inhibitors and either the developmental times or mortality of larvae. Analysis of protein fractions from the seeds indicated that the detrimental effects observed were associated with the globulin fraction (Macedo et al., 1993).

Isolation of vicilins from both *C. maculatus*-resistant and -susceptible seeds and incorporation of these in

artificial seeds showed that purified 7S globulins were responsible for at least part of the detrimental effects seen with resistant seeds (Macedo et al., 1993). This finding prompted us to investigate why vicilins isolated from TVu 2027 seeds conferred resistance to infestation by *C. maculatus*. We found that neither the *C. maculatus*-resistant seeds or vicilins isolated from them had any effect on the development of the bruchid *Z. subfasciatus,* a pest of stored lima bean (*Phaseolus lunatus*) and common bean that can also attack cowpea seeds. Examination of the proteolytic potential of both insects showed that they use, for midgut protein digestion, Cys and aspartic proteolytic enzymes (Silva and Xavier-Filho, 1991). Vicilins from resistant seeds are less susceptible to digestion by enzymes from the midgut of *C. maculatus* than by those of *Z. subfasciatus*. This suggests that at least part of the resistance can be accounted for by these lower rates of hydrolysis (Sales et al., 1992). Additionally, the levels of acidic proteinases in *Z. subfasciatus* were found to be higher than in *C. maculatus* (Silva and Xavier-Filho, 1991). We also found that vicilins isolated from axial tissues of *C. maculatus*-resistant seeds had no effect on development or mortality of the insect, suggesting that these variant 7S globulins are not expressed in these tissues (Melo et al., 1994).

These findings, taken together with results on the stronger binding of vicilins of resistant seeds to chitin structures of the midgut of *C. maculatus*, compared with those of the *Z. subfasciatus* midgut, could satisfactorily explain the detrimental effects of TVu 2,027 seeds (Sales et al., 1996; Firmino et al., 1996). The effects observed with variant vicilins from cowpea and other legumes on *C. maculatus* could then be the result of a reduced availability of amino acids necessary for growth and development of the larvae (Firmino et al., 1996). In Figure 1 we summarize some of our findings on the action of cowpea resistant vicilins on *C. maculatus* and *Z. subfasciatus*.

We also showed that vicilins isolated from seeds of several other legumes such as adzuki bean (*Vigna angularis*), jack bean (*Canavalia ensiformis*), soybean (*Glycine max*), common bean, and lima bean strongly bind to chitin (Yunes et al., 1998). Vicilins from these distantly related species showed a highly detrimental effect on larval development of *C. maculatus*.

VICILINS EXPRESSED IN THE SEED COAT OF LEGUMES MAY HAVE BEEN IMPORTANT FOR THE DISCRIMINATION OF HOST SEEDS BY BRUCHIDS

The seed coat is formed by the integument, maternal tissues surrounding the ovule that were active in the transport of photosynthate to the growing ovary. After seed filling and desiccation the chemical composition of the testa apparently does not reflect that of the tissues that were once active in sugar transport. It is only recently that individual proteins have been reported as being present in the dry seed coat. Among these are enzymes like peroxidases, which perform a role in the deposition of coat pigments, enzymes associated with sugar transport like Suc-P synthase, Suc synthase, basic and acidic invertases,

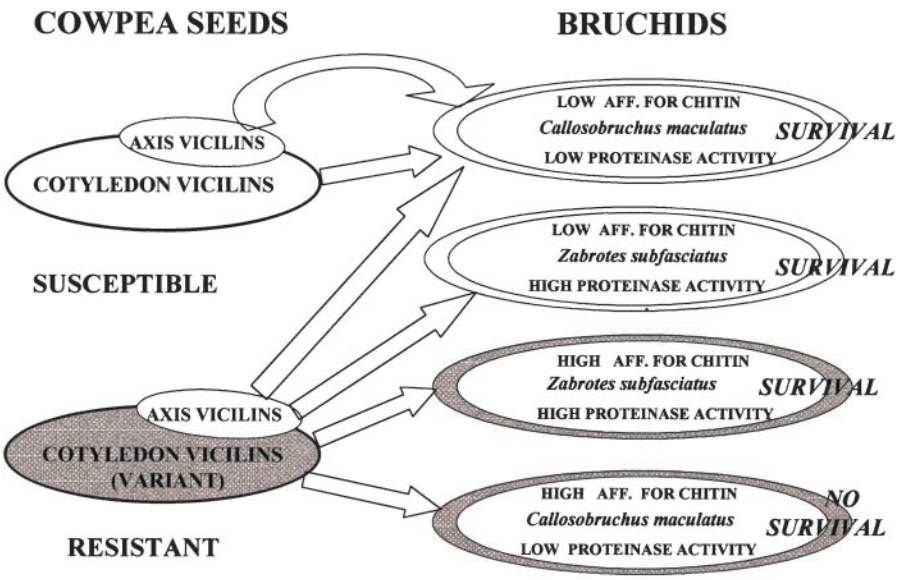

Figure 1. Schematic representation of the action of ingested cowpea vicilins on the bruchids *C. maculatus* and *Z. subfasciatus*. Arrows indicate that isolated vicilins from cotyledon and axial tissues of *C. maculatus*-susceptible and -resistant seeds associate with chitin containing structures of the midgut of the bruchids. The high affinity for chitin of variant vicilins from the cotyledons (and not the axis) of resistant seeds possibly hampers absorption of nutrients through the peritrophic matrix (or equivalent structure). In addition, hydrolysis of vicilins is reduced due to the low level of proteolytic activity found in the midgut of *C. maculatus*. Taken together these factors seem to account for larval mortality of this insect feeding on resistant cowpea seeds.

α-galactosidase, and cell respiration associated enzymes, such as Fru-1,6-biphosphate, aldolase, malate dehydrogenase, Glc-6-P dehydrogenase (Weber et al., 1997). The presence of the sugar transport related hormone insulin and a fragment of its receptor in dry seed coats of jack bean has been shown recently (Oliveira et al., 1999a).

The presence of proteins once thought to be embryo specific, like legumins and vicilins, has also been reported in seed coats of broad bean (*Vicia faba*; Borisjuk et al., 1995). No role for globulins in maternal tissues has been suggested apart from the obvious one of a transitory reserve of amino acids since their expression was found to be transient (Borisjuk et al., 1995). We have found in the dry seed coats of lima bean, common bean, and jack bean a high level of proteins constituted mainly by vicilin polypeptides (Oliveira et al., 1999b). The high levels of vicilins found in the dry testa of these seeds are enough to deter development of first instar larvae of *C. maculatus*.

The interactions between bruchids and legumes are complex and have led to adaptive mechanisms enabling the insects to reproduce and develop on their host plants and adaptations of the plant, limiting losses by the insects. Most bruchids are specialists developing on a limited number of leguminous species. When oviposition occurs on a pod, postembryonic development will occur only if the larva is capable of eating, assimilating, or detoxifying secondary compounds of the seeds. The ability of bruchid larvae to detoxify these compounds is the major cause of host specificity (Janzen, 1981). Due to adaptations developed by bruchids in the presence of their host plants, seed destruction is often very high. Plant defenses leading to reduced losses and retention of reproductive potential are highly varied. The mechanical or chemical barriers produced by the testa or pod pericarp have been considered one of the most important defenses of the plant against bruchids, because they cause high mortality during larval penetration; the production of toxic compounds inside cotyledons may cause high mortality during larval development (Janzen, 1981).

The presence of a *C. maculatus*-detrimental protein in such high levels in the testa of legumes may have had a fundamental evolutionary role allowing the discrimination of non-host seeds by bruchids.

ARCS BELONG TO THE LECTIN-LIKE DEFENSE PROTEIN FAMILY IN BEANS

Until recently, Arcs were thought to be restricted to wild accessions of common bean, but Arc-like sequences were obtained from *Phaseolus acutifolius*, suggesting that they may also be present in other *Phaseolus* spp. (Mirkov et al., 1994). Up to now, seven Arc allelic variants (Arc-1 to -7), which are codominantly inherited, have been described.

Amino acid sequence comparison of Arcs with other seed proteins shows that they belong to the bean defense protein family that includes PHA-L and PHA-E and αAIs. Although these proteins show significant sequence identity (45%–85%) and have similar tertiary structure, they differ in their biochemical properties in the glycosylation patterns, sugar-binding specificities, and quaternary structures. Among Arc variants, they also present different quaternary structures; whereas Arc-2 variant is a dimeric protein, Arc-3 and Arc-4 variants have been reported to be tetrameric proteins, and Arc-1 contains both dimeric and tetrameric forms (Mourey et al., 1997).

The first crystal structure obtained was that of the Arc-5a monomer at 2.7 Å resolution (Hamelryck et al., 1996). The overall structure of Arc-5a is similar to those of the solved legume lectins structures and consists of a flat six-strand β-sheet called the back sheet, packed against a curved seven-strand β-sheet called the front sheet (Hamelryck et al., 1996). In the case of Arc-1, a dimeric structure was obtained at 1.9 Å resolution (Mourey et al., 1998), and its conformation was shown to resemble the canonical dimer conserved among various legume lectins. Superimposition of Arc-5a with the Arc-1 dimer revealed that the Asp-14 and Lys-16 (spatially equivalent to Asn-15 in Arc-1) of one monomer of Arc-5a would clash with the Trp-197 of the other subunit, and the presence of one additional residue inserted (Asp) in the loop 10 to 15 of Arc-5 would probably prevent the formation of an Arc-1-like dimer. This steric conflict could potentially be released by a conformational change of the loop 10 to 15, although accommodation of Lys-16 cannot be easily accounted for (Mourey et al., 1998).

Electrospray mass spectrometry has shown that the Arc-5 variants present different degrees of glycosylation with Arc-5a containing two glycan chains, Arc-5b one glycan chain, and Arc-5c no glycan component (Goossens et al., 1994). Although the presence of glycans has been considered to prevent dimer formation of GS4 and EcorL lectins (Shaanan et al., 1991), it seems that there is no evidence for a correlation between oligomerization and glycosylation states in the case of Arc-5 proteins, since the positions of the three potential glycosylation sites do not exclude the possibility of dimer formation. The contact surfaces between the possible dimers in the crystal of Arc-5a are of comparable size and all resemble typical crystal packing contacts rather than subunit interfaces. Nevertheless, no symmetric dimer is present in the crystal (Hamelryck et al., 1996).

Recently, the *arc-5III* gene that encodes Arc-5c was cloned (Gerhardt et al., 2000), and a molecular model was constructed based on the Arc-5a structure. A comparison of the dimer interface between the PHA-L and Arc-1 structures and the corresponding region of the Arc-5c model, shows that the presence of charged residues (Asp-14, Lys-16, and mainly Asp-53) diminishes the hydrophobic contact area and

places identical charges close together in the putative Arc-5c dimer (Fig. 2).

These amino acid differences in the Arc-5a, and Arc-5c variants explain their weaker dimeric interaction when compared with Arc-1 and other legume lectins. Moreover, the packing for the formation of two canonical lectin dimers that occurs in PHA-L structure would be impossible to occur in Arc-5, because one of two Ser residues present in the PHA-L dimer-dimer interface (Ser-186) is replaced by Lys-181 in Arc-5a (Hamelryck et al., 1996).

The subunit molecular masses of Arc polypeptides range from 30 to 42 kD depending on the variant and show different electrophoretic mobilities from both phaseolin and PHA proteins (Acosta-Gallegos et al., 1998). Arcs are glycoproteins and oligosaccharide chains observed to belong to the complex-and high-Man-type *N*-glycans. Mass spectrometry studies of legume lectins have shown that they undergo extensive post-translational proteolysis at their C termini. In Arc-5a, seven or more residues were clipped from its C terminus (Young et al., 1999).

Arc seed proteins have been shown to have an inhibitory effect on the larval development of the Mexican bean weevil, *Z. subfasciatus* (Osborn et al., 1988; Cardona et al., 1990), and although some wild bean accessions containing Arc are highly resistant to the bean weevil (*Acanthoscelides obtectus*), Arcs appear to have no effect on this insect (Osborn et al., 1988).

Until recently, the majority of Arc studies consisted of protein characterization, bioassays and cDNA cloning. After the crystallization of two Arc proteins (Arc-1 and Arc-5) and the description of sugar-binding properties of Arc-1, a new challenge was presented: What is the mode of action of this storage protein? Recent results can provide some insights to these questions.

UPTAKE OF ARC INTO THE HEMOLYMPH OF BRUCHID LARVAE

Arcs have been shown to confer resistance against bruchid beetles and in vitro results have suggested that higher resistance levels could be achieved by increasing the concentration of Arc (Osborn et al., 1988). Different levels of resistance were found for the different well characterized Arc variants, of which Arc-5 and Arc-1 exhibit the highest levels of resistance to the bean bruchid pest, *Z. subfasciatus* (Cardona et al., 1990). Resistance is evidenced by reduction and delay in adult emergence, usually resulting in a prolongation of the life cycle. Larval mortality occurs by an unknown mechanism and larvae die during the first and second instars.

Despite sequence homology with lectins, Arcs are only weak lectins, and the occurrence of a complex oligosaccharide-binding site has been hypothesized. The sugar binding activity exhibited by Arc-1 toward complex glycans significantly differs from those of PHA-L and PHA-E. In legume lectins, the monosaccharide-binding sites involve a conserved core of four major loop segments at a well-defined site in the surface of each monomer, in which one of the four loops defines the sugar-binding specificity. Arc-1 markedly differs from lectins by substitutions and/or deletions of essential amino acid residues involved in the molecular recognition for metal and sugar binding in these loops, displaying impairment on the monosaccharide binding (Fabre et al., 1998; Mourey et al., 1998). These findings may probably explain why Arcs do not bind simple sugar and sugar derivatives, and, consequently, their weak hemagglutinating properties toward human and rabbit blood cells (Mourey et al., 1998).

Although the insecticidal properties of Arc variants against bruchid pests have been demonstrated, their precise mechanism of action is still unknown: Arc could be toxic as was postulated by Osborn et al. (1988), or it could be indigestible and induce larval starvation (Minney et al., 1990). The entire toxicity of Arc cannot be completely accounted for solely by their resistance to proteases, since the Arc-1 dimer was as insensitive to proteolysis as the two lectins, PHA-L and PHA-E, which are apparently devoid of any toxicity toward *Z. subfasciatus* larvae. It was also proposed that the Asn-linked glycans of Arc-1, which

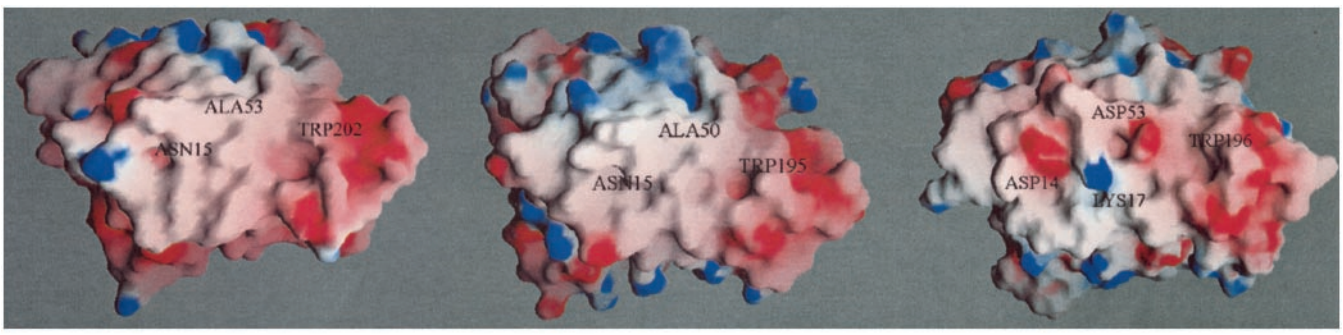

Figure 2. Interdimeric surface charge distribution of PHA-L (left), Arc-1 (center), and Arc-5C (right). Blue, white, and red regions correspond to positive-, neutral-, and negative-charge residues.

are responsible for its binding to Man-specific lectins, known to occur in various insects, may account for its toxicity (Fabre et al., 1998). Thus, it could be postulated, by analogy with PHA proteins, that the toxic properties of Arcs might be related to their recognition and interaction with glycoproteins or other constituents somewhere in the gut of the insect. If Arcs behave as lectins, then two types of interactions could be possible: binding of Arcs to glycoconjugates exposed on the epithelial cells along the digestive tract or to glycosylated digestive enzymes.

To understand the effect of Arc on the insect larvae and why Arc is an insecticidal factor for *Z. subfasciatus* but not for *A. obtectus*, we conducted a study of the effects of dietary Arc on larval gut structure and the distribution of Arc in larval tissues. We showed that Arc-1 had deleterious effects, disrupting the epithelial structure in some regions on the midgut of *Z. subfasciatus*, but not in the midgut of *A. obtectus* larvae (Paes et al., 2000). As immunolocalization shows, Arc protein is abundant in the gut lumen and in cytoplasmic vesicles of the gut lining cells in both *A. obtectus* and *Z. subfasciatus* (Fig. 3, A and B). However, the release of Arc from the gut lumen and its presence in some regions of the hemolymph was found only in *Z. subfasciatus*, indicating that Arc can traverse the cells that line the midgut (Fig. 3B).

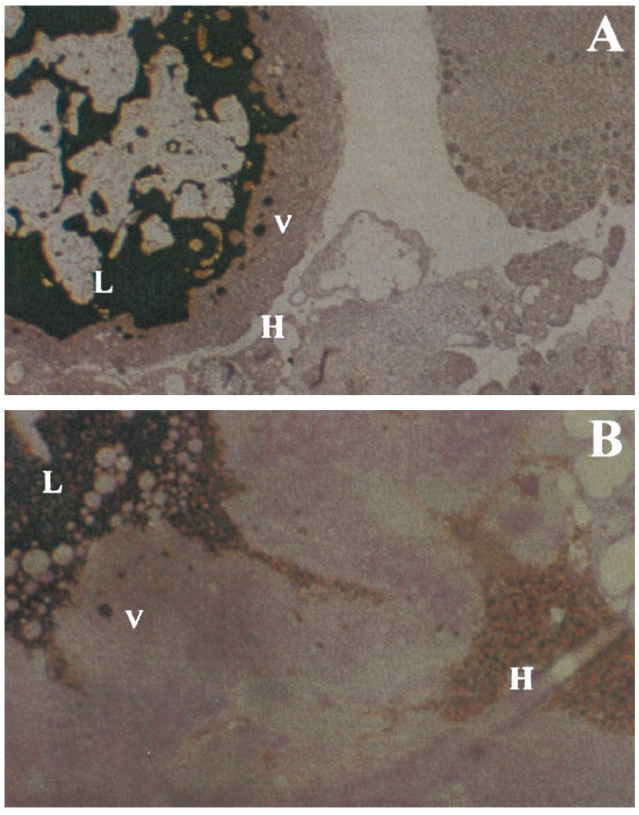

Figure 3. Immunohistochemical detection of Arc protein in the midgut lumen (L), cellular vesicles (V), and hemolymph (H) of *A. obtectus* (A) and *Z. subfasciatus* (B) larvae. Larval tissue was immunolabeled with antibody (anti-Arc-1), and the distribution of specifically bound primary antibody was detected using antibody conjugated with colloidal gold with signal amplified by silver enhancement.

The major difference between the Arc-1 sensitive and resistant bruchid larvae appears to be the presence of the protein in the hemolymph of the sensitive species. However, the mechanism of internalization of Arc-1 through the epithelium remains unclear. The disruption of epithelial gut structure in some regions in *Z. subfasciatus* might permit the passage of Arc-1 into the hemolymph, but it is not clear whether the presence of Arc-1 there contributes to poor larval development or is simply the result of epithelial disruption. Due to the intrinsic specificity of Arc-1 toward complex glycans, Arc could bind to sites or receptors on the midgut structure, but this has yet to be demonstrated. We are presently looking for receptors in *Z. subfasciatus* larval preparations. Such receptors could be located on the plasma membranes of the microvilli of epithelial cells, in the peritrophic matrix, or in the hemolymph. Mourey et al. (1998) recently suggested on the basis of binding experiments of Arc-1 and various glycoproteins (fetuin, thyroglobulin) that the protein may have an extended carbohydrate-binding site in the vicinity of the unreactive sugar-binding pocket.

CONCLUDING REMARKS

In this *Update*, we have described work on legume seed storage proteins that have been recruited through co-evolutionary processes as defense proteins toward bruchid insects. Much work is necessary in order to unravel these processes and the steps that were necessary to achieve the biological activities now apparent in variant vicilins and Arcs as well as the mechanisms that underlie their action. The use of these host-specific defense proteins for conferring resistance to other plants through the development of biotechnological means should also be strongly stimulated.

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