Analysis of bruchid resistance in the wild common bean accession G02771: no evidence for insecticidal activity of arcelin 5

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Abstract

Arcelins are abundant seed storage proteins thought to be implicated in the resistance of wild Phaseolus vulgaris (L.) genotypes against Zabrotes subfasciatus (Boheman), an important storage insect pest of common bean. Here, the insecticidal activity of the arcelin-5 variant that is present in the highly resistant P. vulgaris accession G02771 was investigated. No correlation could be established between the presence of arcelin 5 and the insecticidal effects observed in G02771 seeds. Insect feeding assays with artificial seeds into which purified arcelin-5 protein was incorporated and with transgenic P. acutifolius (A. Gray) seeds in which the arcelin-5 genes were expressed, showed that the presence of arcelin-5 proteins, even at elevated levels, was not sufficient to achieve adequate resistance against Z. subfasciatus. The same might apply to other arcelin variants. Nevertheless, as resistance is clearly closely linked to the presence of the arcelin-1 or arcelin-5 locus, arcelins remain useful markers in breeding programmes aimed at introducing high levels of resistance to Z. subfasciatus in P. vulgaris cultivars.

Key words: Arcelin, bruchid resistance, Mexican bean weevil, Phaseolus, Zabrotes subfasciatus.

Introduction

In the continuous search for new plant genes that confer resistance against insects, some interesting candidates have been found in the gene family of lectins and ‘lectin-like’ proteins of the common bean (Phaseolus vulgaris [L.]) (Chrispeels, 1997). Different members of this family are thought to be toxic towards insects of the coleopteran family Bruchidae, which commonly infest leguminous seeds and cause important (post) harvest losses. The P. vulgaris lectin gene family consists of the true lectin phytohemagglutinin (E and L variant) and the lectin-like proteins, arcelin and a-amylase inhibitor. Phytohemagglutinin (PHA) was the first member of this family to which insecticidal properties were ascribed (Janzen et al., 1976). Later on, the deleterious effects on the cowpea weevil (Callosobruchus maculatus [Fabricius]) were shown to be due not to PHA, but to a contamination of the PHA preparation with the bean a-amylase inhibitor (aAI) (Huesing et al., 1991). Some promising results were subsequently obtained with the latter protein. In experiments with artificial diets as well as in feeding trials with seeds of transgenic leguminous plants, aAI displayed relatively high toxicity levels, not only to some bruchid species but also to members of other insect families (Ishimoto and Kitamura, 1989; Pueyo et al., 1995; Shade et al., 1994; Schroeder et al., 1995; Ishimoto et al., 1996). Although the bean aAI may protect P. vulgaris seeds...
from infestation by some bruchids, it is ineffective against the two major bruchid pests of common bean, the bean weevil (Acanthoscelides obtectus [Say]) and the Mexican bean weevil (Zabrotes subfasciatus [Bohemian]). Interestingly, novel α1 variants with insecticidal effects on Z. subfasciatus were isolated from some wild Phaseolus genotypes that were resistant against Z. subfasciatus (Ishimoto and Kitamura, 1993; Ishimoto and Chrispeels, 1996). Whether these α1s are good candidates to achieve resistance against Z. subfasciatus in common bean is still unclear. Loss of resistance in breeding lines was observed although they still showed high levels of Z. subfasciatus α-amylase inhibition (Goossens et al., 1996).

Cultivated common bean genotypes are generally not resistant to the bean weevil or the Mexican bean weevil. In contrast, high levels of resistance to the Mexican bean weevil were found in wild P. vulgaris populations of Mexican origin (Schoonhoven et al., 1983). Osborn et al. proposed that arcelins are associated with the resistance phenotype (Osborn et al., 1988). Arcelins are abundant, lectin-like seed storage proteins that are present in wild P. vulgaris accessions resistant to Z. subfasciatus, but not in cultivated common beans. So far, seven allelic arcelin variants have been identified (Osborn et al., 1986; Lioi and Bollini, 1989; Santino et al., 1991; Acosta-Gallegos et al., 1998), designated arcelin 1 to arcelin 7, of which variants 1 and 5 seem to be the most promising candidates to provide bruchid resistance in leguminous crops. Wild P. vulgaris accessions containing one of these two variants showed the highest resistance levels to the Mexican bean weevil (Cardona et al., 1990) and more importantly, when arcelin-containing accessions were used in a breeding programme (Kornegay et al., 1993), high resistance levels were only maintained in lines generated from crosses with arcelin-1 or arcelin-5 parents. Insecticidal activity of arcelin 1 was also observed in feeding experiments with artificial seeds (Osborn et al., 1988). High levels (10% w/w) of purified arcelin-1 proteins reduced significantly the larval development of Z. subfasciatus. Lower levels however (5% w/w), had no significant effect.

Compared to the arcelin-1 variant (Romero-Andreas et al., 1986), arcelin 5 was discovered later (Lioi and Bollini, 1989) and was consequently not included in the first series of breeding experiments (Cardona et al., 1990) and artificial seed assays (Osborn et al., 1988). Arcelin 5 is present in the wild G02771 accession and consists of three polypeptides (Arc5a, Arc5b and Arc5c), which are, in the native state, organized as monomers or dimers (Goossens et al., 1994; Hamelryck et al., 1996). Arcelin 5 is encoded by two genes: arc5-I that codes for Arc5a and arc5-II that codes for both Arc5b and Arc5c, with Arc5c representing a non-glycosylated isoform of Arc5b (Goossens et al., 1994, 1999). The sequence identity between the coding regions of arc5-I and arc5-II is more than 98%.

Here, the role of arcelin 5 in the resistance phenotype of the G02771 accession towards Z. subfasciatus is investigated. The insecticidal activity of the arcelin-5 protein was assessed in vitro; insect feeding assays indicated that the presence of arcelin 5 and the antibiosis effects of G02771 seeds against the Mexican bean weevil could not clearly be correlated. To understand the mechanism of toxicity in G02771 seeds, microscopic studies of bruchid larval sections were undertaken.

**Materials and methods**

**Plant material**

The Phaseolus lines used were the P. vulgaris cultivar Ica Pijao, the P. vulgaris wild genotypes G12882 (containing arcelin 1), G12952 (containing arcelin 4), and G02771 (containing arcelin 5), and the P. acutifolius A. Gray wild genotypes N1576 (NI for collection number of the Belgian National Botanical Garden, Meise, Belgium), G40168, and G40199. The P. vulgaris cultivar Ica Pijao and the P. acutifolius lines N1576 and G40168 are susceptible to the Mexican bean weevil, whereas all arcelin-containing P. vulgaris lines and the P. acutifolius line G40199 are highly resistant against this insect.

**Transformation of P. acutifolius with arcelin-5 genes**

P. acutifolius (genotype N1576), was transformed as described (Dillen et al., 1997). Co-cultivation was carried out with the Agrobacterium tumefaciens strain C58C1Ri8 containing the helper plasmid pMP90 (Koncz and Schell, 1986) and harbouring the binary plasmids pATARC3-B1b or pATARC3-B52b (Goossens et al., 1999). pATARC3-B1b contains between the T-DNA borders, in addition to the marker genes coding for neomycin phosphotransferase II (nptII) and β-glucuronidase (uidA), a BamHI fragment of the arc5-I gene (Goossens et al., 1995). pATARC3-B52b is identical to pATARC3-B1b except that the arc5-I-coding region is replaced by that of arc5-II.

**Detection and quantification of arcelin-5 proteins in transgenic P. acutifolius seeds**

Crude seed protein samples were obtained by two successive extractions of ground cotyledon tissue in 10 mM NaCl, 50 mM glycine, pH 2.4 for 30 min at room temperature under continuous shaking. After centrifugation for 10 min at 20000 g, the pellet was removed and supernatants were pooled. Protein concentrations of the crude extracts were determined by measuring UV absorbance at 280 nm. Proteins were separated by sodium dodecyl sulphate/polyacrylamide gel electrophoresis as described previously (Hames and Rickwood, 1990). Arcelin-5 proteins in transgenic P. acutifolius seeds were visualized and detected by protein gel blot analysis and expression levels were estimated (as a percentage of total extractable seed protein) by indirect ELISA (as described by Harlow and Lane, 1988) using a rabbit polyclonal anti-arcelin-5 antiserum. Arcelin-5 proteins purified from the P. vulgaris G02771 accession, mixed with total seed proteins of the non-transgenic P. acutifolius accession N1576, were used to construct a standard curve.

**Preparation of ‘artificial’ seeds**

Arcelin 5 (mixture of Arc5a, Arc5b and Arc5c) was purified on a large scale from seeds of the P. vulgaris G02771 accession using the procedure described previously (Goossens et al., 1994). The purified protein was precipitated with an excess
(NH$_4$)$_2$SO$_4$, resuspended in distilled water, dialysed against several changes of distilled H$_2$O to remove the remaining (NH$_4$)$_2$SO$_4$ crystals, and lyophilized before incorporation into artificial seeds.

Artificial seeds containing various levels of arcelin-5 proteins (% w/w) were prepared according to the technique devised previously (Shade et al., 1986) for the cowpea weevil. Beans (Ica Pijao) were soaked, the testae were removed, and the flour was milled (<250 µm) and dried. The flour, with or without the addition of purified seed proteins, was mixed with water, reconstituted in moulds, lyophilized, and then rehydrated (to approximately 13% relative humidity) at room temperature. Artificial seeds were coated with a thin layer of gelatin, and infested as if they were intact seeds.

**Insect rearing and feeding tests**

Techniques to maintain insect cultures and to test accessions and breeding lines were identical to those described previously (Schoonhoven and Cardona, 1982; Cardona et al., 1989). All experiments were done at 27 °C and 70% relative humidity in a controlled environment chamber.

Artificial seeds were tested in two replicates, each replicate consisting of five seeds infested with five Mexican bean weevil eggs per seed. Intact transgenic P. acutifolius seeds were tested in three replicates using four pairs of Mexican bean weevil adults to infest 15–20 seeds per replicate. Development on artificial seeds and transgenic P. acutifolius seeds was compared with that on intact, wild-type P. vulgaris or P. acutifolius seeds based on days to adult emergence and percentage emergence. Percentage emergence and days to adult emergence were submitted to analysis of variance; treatment means were separated using Duncan’s Multiple Range Test (SAS Institute, 1985). Percentage of emergence was transformed to arcsin square root. Non-transformed data are shown.

**Immunolocalization of arcelin 5 and phaseolin in Z. subfasciatus and A. obtectus larvae**

Samples were prepared for light microscopy as follows. Whole Z. subfasciatus or A. obtectus larvae, reared on Ica Pijao or G02771 seeds, were fixed at 4 °C for 24 h in a solution of 2.5% paraformaldehyde, 0.5% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.2). After three washes for 2 h in 0.1 M Na-cacodylate (pH 7.2) at 4 °C, samples were dehydrated through graded ethanol series. The 30%, 50%, 70%, and 95% ethanol steps (2 h each) and a final 95% ethanol step (24 h) were performed at 4 °C. The samples were embedded by incubating them successively in a 1/1 (v/v) ratio of 95% ethanol/LR White (hard grade; London Resin Co., Basingstoke, UK) (for 24 h) and in pure resin (for 48 h, three changes) at 4 °C. A 6 h incubation step using fresh LR White at 4 °C was performed before the samples were placed in gelatin capsules containing fresh nitrogen fluxed resin. Polymerization was performed by UV illumination at −20 °C for 48 h and at room temperature for 24 h. Capsules were then placed at 60 °C for 24 h to ensure complete polymerization of resin.

Semi-thin sections (1–3 µm) were made using a 2050 microtome (Reichert-Jung, Nüßloch, Germany), collected on electrostatically charged microscope slides (BDH, Poole, UK) and immunostained using the AuroProbe®One and IntenSE® reagents (Amersham, Aylesbury, UK) for immunogold silver staining. Rabbit polyclonal anti-arcelin-5 or anti-phaseolin antiserum was used as primary antibody. Sections were examined by light microscopy (Leitz, Wetzlar, Germany).

**Results**

**Effect of purified arcelin-5 proteins on larval development of Z. subfasciatus**

In a first way to assess the effect of arcelin 5 on bruchid development, artificial bean seeds were produced that contained purified arcelin-5 proteins (the mixture of Arc5a, Arc5b and Arc5c) and tested for resistance to Z. subfasciatus. Results are presented in Fig. 1. Intact seeds and artificial seeds of Ica Pijao and G02771 were included as controls. Although the absolute values for days to adult emergence and percentage emergence for artificial seeds were slightly different from those for intact seeds, resistant and susceptible phenotypes were easily distinguishable. Data for intact Ica Pijao and G02771 seeds were not included in Fig. 1, but were similar to those shown in Table 1. Ica Pijao is susceptible to bruchid infestation whereas G02771 is highly resistant.

In this experiment different levels of arcelin-5 protein (from 1–10% w/w) were added to the flour of the susceptible cultivar Ica Pijao and tested for insecticidal activity. The level of 10% (w/w) represents the approximate physiological concentration of arcelin 5 in seeds of the wild G02771 accession. At the lower levels tested (up to 5% w/w), arcelin 5 had little or no effect on larval development. At higher levels, arcelin 5 caused an increase in the days to adult emergence of 29% (with 7.5% Arc5) and 48% (with 10% Arc5), and a reduction in adult emergence of 23% (with either 7.5% or 10% Arc5), compared to the

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![Fig. 1. Effect of purified arcelin-5 proteins on larval development of Z. subfasciatus](image-url)
susceptible control. Nevertheless, even with artificial seeds containing 10% Arc5, the increase in the days to adult emergence and the reduction in adult emergence was much lower than with artificial seeds prepared from non-transformed G02771 flour (Fig. 1).

Resistance levels of transgenic P. acutifolius lines to Z. subfasciatus

Another way to analyse the involvement of arcelin 5 in bruchid resistance was to determine whether resistance was correlated to the genetic transfer of the arcelin-5 allele. Therefore, the arcelin-5 genes were introduced into P. acutifolius and the transgenic progeny was tested for bruchid resistance. Transgenic plants containing the arc5-I gene were shown to produce high levels of Arc5a (Goossens et al., 1999) (Fig. 2, lane A), whereas plants in which the arc5-II gene was introduced synthesized Arc5b and Arc5c (Goossens et al., 1999) (Fig. 2, lane B).

In the insect trials (Table 1), seeds of a fully susceptible (G04168) and a resistant (G40199) P. acutifolius line, as well as seeds of the wild-type (non-transformed) P. acutifolius line NI576, the resistant P. vulgaris genotype G02771, and the susceptible P. vulgaris cultivar Ica Pijao were included as controls. The wild-type P. acutifolius line NI576 showed intermediate levels of susceptibility. Therefore, data obtained with seeds of transgenic lines, derived from this NI576 line should be interpreted with caution. Different environmental conditions during plant and/or seed development can influence levels of resistance significantly: intermediately susceptible lines can become fully susceptible depending on the origin of the seed. To minimize this possible influence, seeds of NI576 and NI576-derived lines assayed for bruchid resistance and included in Table 1 were harvested from homozygous plants, cultivated at the same time in the greenhouse. Nevertheless, a difference in resistance levels was observed for the wild-type NI576 line and the NI576-44.x.0 line, a progeny line from transgenic plant NI576-44.x that does not contain a transgene locus. The reason for the difference between these two lines is unclear.

The main result from the insect assays is that none of the transgenic P. acutifolius lines displayed levels of resistance comparable to those of G04199, the resistant P. acutifolius control or G02771, the resistant P. vulgaris line (Table 1). Moreover, most lines that produced arcelin 5 were as susceptible as the wild-type NI576 line, even when arcelin 5 was present at levels up to 25% of total seed protein. The slight increase in resistance observed in lines NI576-8.1.22 and NI576-55.1.10 was most probably not related to the presence of the arcelin. In another trial with seeds harvested at another period, NI576-8.1.22 seeds were as susceptible as seeds from the wild-type NI576 line and as seeds from NI576-8.1.0, a progeny line in which the transgene was not present anymore (data not shown).

To determine whether the presence of all arcelin-5 isoforms could confer resistance, NI576 lines synthesizing all isoforms (Fig. 2, lane CR) were constructed by crossing an arc5-I (NI576-8.1.22) with arc5-II lines (NI576-44.x.59 or NI576-55.1.10). Insect trials showed similar susceptibility levels for these lines as observed for lines expressing arc5-I or arc5-II only (data not shown).

Table 1. Effect of transgenic P. acutifolius genotypes on larval development of Z. subfasciatus

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Arc5a (%)</th>
<th>Emergence (%)</th>
<th>Days to adult emergence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transgenic P. acutifolius lines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NI576-44.x.0</td>
<td>N</td>
<td>58.1 fg</td>
<td>39.4 de</td>
</tr>
<tr>
<td>NI576-8.1.22</td>
<td>23.7 A</td>
<td>58.0 fg</td>
<td>39.8 d</td>
</tr>
<tr>
<td>NI576-27.6.18</td>
<td>16.2 A</td>
<td>70.2 ef</td>
<td>44.6 c</td>
</tr>
<tr>
<td>NI576-44.x.59</td>
<td>25.4 B</td>
<td>75.8 ede</td>
<td>44.8 e</td>
</tr>
<tr>
<td>NI576-44.k4.1.2</td>
<td>15.7 B</td>
<td>70.9 def</td>
<td>42.2 cd</td>
</tr>
<tr>
<td>NI576-55.1.10</td>
<td>20–30 B</td>
<td>46.1 g</td>
<td>46.1 c</td>
</tr>
<tr>
<td>Wild-type P. acutifolius lines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NI576</td>
<td>N</td>
<td>74.8 ede</td>
<td>42.5 cd</td>
</tr>
<tr>
<td>G40168</td>
<td>N</td>
<td>83.7 bcd</td>
<td>35.3 ef</td>
</tr>
<tr>
<td>G40199</td>
<td>N</td>
<td>1.2 h</td>
<td>59.0 b</td>
</tr>
<tr>
<td>Wild-type P. vulgaris lines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G02771</td>
<td>35–40 A+B</td>
<td>4.4 h</td>
<td>70.9 a</td>
</tr>
<tr>
<td>Ica Pijao</td>
<td>N</td>
<td>97.4 a</td>
<td>31.4 f</td>
</tr>
</tbody>
</table>

Means of three replicates, each replicate consisting of 15–20 seeds infested with four pairs of Z. subfasciatus adults.

* Level of arcelin as percentage of total seed protein of transgenic P. acutifolius seeds. (A) Arc5a; (B) Arc5b and Arc5c; (N) no arcelin-5 proteins present.

b Means followed by the same letter are not significantly different (P<0.05) (Duncan’s multiple range test; SAS Institute, 1985).

c Seeds of progeny of the transgenic line NI576-44, which do not contain the arc5-II transgene.

d This line contains more than one transgenic locus segregating in the present seed batch and therefore an estimated range of arcelin-5 expression levels is indicated.
Immunolocalization of P. vulgaris seed storage proteins in Z. subfasciatus larval sections

To elucidate the mechanism of toxicity in G02771 seeds and visualize possible insecticidal effects of G02771 seed compounds, microscopic studies of bruchid larval sections and immunolocalization of P. vulgaris seed storage proteins in such sections were undertaken. Z. subfasciatus larvae were reared either on G02771 or on Ica Pijao seeds. Immunolocalization was performed on sections of first, second or third instar larvae with a rabbit polyclonal anti-arcelin-5 antiserum or, as a control, with a rabbit polyclonal antiserum against phaseolin, the normal common bean seed storage protein. Larvae of A. obtectus, of which the development is not or hardly affected when fed on G02771 seeds, were submitted to the same treatment. Antisera were obtained from rabbits injected with chemically deglycosylated (as described by Edge et al., 1981) mature arcelin 5 or denatured, but glycosylated, mature phaseolin from the P. vulgaris cultivar Greensleeves. Specificity of the antisera is demonstrated in Fig. 3. Immunostaining of the sections was also performed with pre-immune sera of rabbits used to generate the anti-arcelin-5 antiserum as a negative control (data not shown).

Larvae of Z. subfasciatus, when fed on Ica Pijao seeds, did not show any specific staining for arcelin 5 (Fig. 4A) nor for phaseolin (Fig. 4B), apart from the gut content, which consists of seed pieces containing more or less intact seed proteins. This result was expected because larvae that do not suffer growth defects should have normally functioning guts, leading to an efficient digestion of Phaseolus seed proteins. In contrast, results obtained for Z. subfasciatus larvae fed on G02771 seeds (Fig. 4C–H) indicated a general defect in the structural integrity and/or functionality of the larval digestive system. Surprisingly, in these larvae the arcelin-5 protein was present over the entire larval section (Fig. 4C, E, first and second instar larvae, respectively), in all larval tissues and organs. A similar distribution pattern was also observed for the phaseolin protein in these larvae (Fig. 4D, F, first and second instar larvae, respectively). Because the antisera used were polyclonal and, in the case of the anti-arcelin-5 antiserum, also recognized arcelin-5 degradation products (A Goossens, unpublished results), intact or partially digested seed storage proteins could not be distinguished.

The arcelin-5 protein was also localized over the entire section of first and second instar larvae of A. obtectus fed on G02771 seeds (Fig. 4I). To a lesser extent, this was also the case for phaseolin in first instar larvae (data not shown), but not in second instar larvae (Fig. 4J). This observation was unexpected, because the development of this bruchid seems not, or hardly, affected by a diet of G02771 seeds (Cardona et al., 1990). Insect assays revealed only a slight effect of the G02771 diet on A. obtectus larval development, which finally caused some growth retardation (37.3 d and 32.3 d to adult emergence on G02771 and Ica Pijao seeds, respectively), but hardly affected the number of adults emerging (85.2% on G02771 seeds versus 95.3% on Ica Pijao seeds; A Goossens and C Cardona, unpublished results). Cardona et al. found that Z. subfasciatus larvae fed on arcelin-containing seeds were most affected in the early developmental stages, i.e. first and early second instar showed highest mortality rates (Cardona et al., 1989). Most larval individuals that reached the third instar phase could pupate and emerge as an adult weevil. This observation correlates with the fact that the immunological staining for arcelin 5 as well as for phaseolin decreased significantly, or almost disappeared, in third instar larvae of Z. subfasciatus fed on G02771 seeds (Fig. 4G, H). Immunolocalization studies of A. obtectus larvae in later developmental stages were not performed.

Discussion

In tropical and subtropical countries, the common bean suffers important post-harvest losses caused mainly by the bruchid pests Z. subfasciatus and A. obtectus. High levels of resistance against Z. subfasciatus were found in a number of wild P. vulgaris populations of Mexican origin whereas cultivated P. vulgaris genotypes exhibit no or very low levels of resistance (Schoonhoven et al., 1983). A common feature of these resistant accessions is the presence of a novel seed storage protein, designated arcelin, which represents a third important seed protein fraction in these accessions, in addition to phaseolin and phytohemagglutinin, the two major protein fractions normally present in common bean seeds. In inheritance

Fig. 3. Specificity of the anti-arcelin-5 and anti-phaseolin antisera. Lanes contain crude protein extracts of seeds of the wild P. vulgaris accession G02771 (W) or the cultivar Ica Pijao (C), obtained after extraction with buffer containing 50 mM NaCl, 50 mM glycine, pH 2.4. Panels show a Coomassie blue-stained sodium dodecyl sulphate/polyacrylamide gel electrophoresis (A) and protein gel blot analysis with the anti-arcelin-5 (B) or anti-phaseolin antisera (C). The position of phaseolin and arcelin-5 proteins is indicated with an asterisk and a triangle, respectively. M, marker proteins (molecular mass indicated in kDa).
studies with wild accessions containing arcelin 1 or arcelin 5, resistance to the Mexican bean weevil was found to cosegregate with the arcelin allele (Kornegay et al., 1993), demonstrating that arcelin itself, or a factor linked to it, causes resistance. Further indications that arcelins are involved in the resistance of these wild genotypes were provided by experiments showing that larval development was strongly affected on artificial diets to which high amounts of arcelin-1 proteins were added (Osborn et al., 1988). It should be realized, however, that the composition of such artificial diets differs in several parameters from that of arcelin-containing beans, among which the total protein content, the amount of arcelin as a percentage of total protein, and the arcelin/phaseolin ratio. Moreover, it is difficult to exclude the possibility that not arcelin itself, but a factor contaminating the arcelin preparation, is causing resistance. Therefore, the insecticidal activity of the arcelin-5 variant, present in the wild *P. vulgaris* genotype G02771, and its significance in the resistance against the Mexican bean weevil was investigated in detail.

The ability of the arcelin-5 protein to reduce *Z. subfasciatus* larval development was tested *in vivo* by growing the larvae either on an artificial diet into which high amounts of purified arcelin-5 proteins were incorporated or on transgenic *P. acutifolius* seeds in which the arcelin-5 genes were highly expressed. When incorporated in artificial seeds, arcelin 5 had little or no effect on larval development at the lower levels tested (±5% w/w), whereas at higher levels (±7.5% w/w) arcelin 5 caused some reduction in adult emergence and an increase in the days to adult emergence. But, even at a level corresponding with the physiological concentration of arcelin 5 in seeds of the wild G02771 accession (10%, w/w), the overall level of resistance in the artificial seeds was much lower than in the G02771 seeds or in artificial seeds prepared from G02771 flour.

In an attempt to obtain more conclusive proof of the role of arcelin 5, the arcelin-5 genes were introduced into an insect-susceptible, transformation-competent *P. acutifolius* genotype. In this way, only one new trait, i.e. the presence of arcelin-5 proteins, was introduced into a susceptible line. Because seeds of the G02771 accession contain three closely related arcelin polypeptides (Arc5a, Arc5b and Arc5c) that are encoded by two genes (*arc5-I* and *arc5-II*; Goossens et al., 1994, 1999), transgenic *P. acutifolius* lines expressing all possible arcelin-5 gene
combinations were generated. The resistance levels of transgenic *P. acutifolius* lines, expressing *arc5-I, arc5-II* or both, were significantly lower than those of the *P. vulgaris* accession G02771. Moreover, most transgenic lines were as susceptible as the non-transformed control, even when arcelin 5 was present at a level of up to 25% of total protein, a level associated with high resistance in *P. vulgaris* breeding lines (Kornegay et al., 1993; A Goossens and C Cardona, unpublished results). Although the effect of introduced arcelin genes was assayed in a genetic background (NI576) that is different from that of the recurrent parents employed in backcross breeding programmes, this difference would probably not entirely mask an effect of arcelin 5. Results obtained in this experiment consequently argue against arcelin 5 possessing a significant insecticidal effect on its own.

In view of the above, it appears that the resistance phenotype of the G02771 accession to the Mexican bean weevil is not due to arcelin 5 itself, but to the presence of another factor, genetically closely linked to the *arc5*-5 allele. This is supported by the observation that some breeding lines that highly express arcelin 5 are not resistant to bruchids (C Cardona and A Goossens, unpublished results). The putative arcelin-associated factor may act on its own or in concert with arcelin. An accessory or synergistic effect of arcelin might, for example, be related to its poor digestibility by larval enzymes (Minney et al., 1990).

The nature of the insecticidal factor in G02771 seeds, as well as in seeds of common bean genotypes containing the other arcelin variants, remains consequently unknown. Different *in vitro* experiments (Suzuki et al., 1995; Fory et al., 1996; Quintero, 1997; A Goossens and C Quintero, unpublished results) suggest that the resistance phenotype of G02771 seeds is not based on an inhibitory effect against *Z. subfasciatus* larval proteases and/or α-amylases. This lack of α-amylase and protease inhibitory activity is encountered in most arcelin-containing lines, at least under the experimental conditions used. Inhibitory activity towards *Z. subfasciatus* α-amylases is found only in seeds of arcelin-4-containing lines (such as G12952) (Fory et al., 1996; Ishimoto and Chrispeels, 1996) and is caused by the αA2 gene product. However, Fory et al. also described that the *arc5*/αA2 locus does not suffice to cause the high *Z. subfasciatus* resistance levels of the G12952 line and suggested that genes not residing at this locus contribute strongly to the overall resistance levels of this genotype (Fory et al., 1996). To understand the nature of the toxicity in arcelin-5-containing seeds further, immunostainings for bean seed proteins were performed on *Z. subfasciatus* larvae fed on G02771 seeds. Surprisingly, arcelin 5 and also phaseolin, the normal and non-toxic common bean seed storage proteins, could be detected in all larval tissues and structures, suggesting that a factor present in G02771 seeds interferes with normal functionality or structural integrity of the larval digestive system. Preliminary histological (A Goossens and F Jacobs, unpublished results) and immunolocalization studies at the electron microscopy level (A Goossens and R De Rycke, unpublished results) did not reveal obvious defects at the cellular level. Further research is required to clarify the way in which the putative arcelin-associated factor is involved in disruption of the larval digestive system and whether or not this is a primary effect.

In conclusion, the experiments reported here strongly suggest that the arcelin-5 protein on its own does not provide satisfactory resistance levels to bruchids. In view of these results, it may be worthwhile to reassess the role of other arcelin variants in bruchid resistance. Notwithstanding, arcelins could be part of a general resistance management programme, combining different insecticidal factors to achieve a durable bruchid resistance. Because arcelins show a high biochemical stability, they could add to the effect of other insecticidal products and further impair bruchid development. Also, since in the case of arcelin 1 and arcelin 5 resistance is clearly closely linked to the presence of the arcelin locus, these arcelins remain useful markers to introgress high levels of resistance to *Z. subfasciatus* in *P. vulgaris* cultivars by conventional breeding (Cardona et al., 1992; Padgham et al., 1992).

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