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Transfer of the Arcelin-Phytohaemagglutinin- α Amylase Inhibitor Seed Protein Locus from Tepary bean (*Phaseolus acutifolius* A. Gray) to Common Bean (*P. vulgaris* L.)

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Abstract: *Phaseolus vulgaris* cultivars ICA Pijao, Rojo and 5-593 were crossed to the *P. acutifolius* wild accessions G40199 and an F₂ selection from a cross between G40199 and cultivated brown seeded unnamed tepary accession (designated Brown Tepary). G40199 is highly resistant to the two major bruchid pests of common bean: *Acanthoscelides obtectus* and *Zabrotes subfasciatus*, but the mechanism for resistance is unknown. Interspecific F₁ hybrids with the three common bean parents were generated via., embryo rescue. Recovered hybrids were from ICA-Pijao and 5-593 and were highly sterile and were backcrossed as females to ICA Pijao. Seeds from the BC₁F₂ plants were screened for protein phenotype and the inheritance of seed storage protein profiles contributed by the tepary bean parents. Most of the F₁ hybrids demonstrated introgression of a lectin-like protein of 33 kDa that was found in G40199, but not in the Brown Tepary or common bean lines. This lectin related protein complex was similar to the arcelin (ARL), phytohaemagglutinin (PHA) and α -amylase inhibitor (α -AI) seed storage protein family of *P. acutifolius*. Genomic DNA sequences from wild accession G40199 and the interspecific hybrids revealed a high sequence similarity to ARL2 and α -AI genes of *P. acutifolius*. Because lectin-related proteins of *P. acutifolius* have been associated with strong resistance to bruchids, we hypothesize that these proteins alone or in conjunction with other factors, may contribute to the disputed bruchid resistance mechanism in G40199.

Key words: Lectin-like arcelin, bruchid resistance, embryo rescue, interspecific hybridization, *Phaseolus acutifolius*

INTRODUCTION

In the tropics and subtropics, the bruchids *Zabrotes subfasciatus* (Mexican bean weevil) and *Acanthoscelides obtectus* (common bean weevil) are major pests of beans in storage. Larvae burrow into the seeds to feed and metamorphose from larva to adult within the seed. Adult bruchids cause no direct damage to the beans in storage, but females can lay up to 60 eggs depending on the species and host (Parsons and Credland, 2003). Damage is directly related to the number of larvae that hatch and burrow into the seeds. Weevil damage can reduce quality and yield as much as a 48% (Slumpa and Ampofo, 1991). The average worldwide loss caused by weevil damage ranges from 7-40% of marketable beans.

While, large warehouses use chemical controls to reduce and eliminate infestations, small holder farmers in developing countries usually pursue low-cost, low-technology strategies for control. Farmers reduce their losses by limiting the time that they keep their harvest and

by not producing large quantities of the crop in any one growing season. Breeding for disease and insect resistance can increase yield stability, thereby contributing to a stable supply of dry beans in developing countries. Resistance to bruchids is a simply inherited factor found in wild *P. vulgaris* accessions and tepary beans *P. acutifolius* of the Mesoamerican origin. In particular, high levels of resistance to *Z. subfasciatus* and moderate resistance to *A. obtectus* have been characterized by Cardona *et al.* (1990), Kornegay and Cardona (1991), Kornegay *et al.* (1993), Suzuki *et al.* (1995) and Hartweck *et al.* (1997). Resistance is associated with lectin-like seed storage proteins (LLPs) (Sales *et al.*, 2000), particularly, arcelin (ARL) (Osborn *et al.*, 1986, 1988; Lioi and Bolini, 1989; Minney *et al.*, 1990; Goossens *et al.*, 1994; Santino *et al.*, 1991; Acosta-Gallegos *et al.*, 1998) and alpha amylase inhibitor (α -AI) (Fory *et al.*, 1996; Grossi de Sá *et al.*, 1997). Along with the lectin-phytohaemagglutinin (PHA), genes for these closely related seed storage proteins make up the

complex ARL-PHA- α -AI (APA) locus. Lioli *et al.* (2003) hypothesized that the LLPs evolved from PHA. They provided evidence that multiple duplication events followed by divergence in form and function led to the origin of ARLs and α -AIs. In this way, functional properties of lectins and LLPs have diverged: PHAs bind carbohydrates, α -AIs bind to amylase proteins and the biological activity of ARLs is unclear. All acts act as deterrents to seed predation by insects, mammals and birds.

Seven arcelin-ARL alleles from *P. vulgaris*, have been described by Osborn *et al.* (1986), Lioi and Bollini, (1989), Santino *et al.* (1991) and Acosta-Gallegos *et al.* (1998). More than one arcelin variant may be present in a single accession suggesting that ARLs are not alleles at a locus in the classic sense (Lioi *et al.*, 2003). Rather, there are at least two tightly linked loci that function effectively as a single locus. Accessions that possess the different allelic combinations give varying levels of resistance to the two bruchid species. Some (Arl-1, 2, 4 and 5) confer strong resistance to *Z. subfasciatus*, but weak resistance to *A. obtectus* (Cardona *et al.*, 1990) but others give only weak to moderate resistance to both species. There is none that give strong resistance to both bruchid species.

In determining the effectiveness of the ARL alleles, researchers have been hindered by different alleles being in different genetic backgrounds. Hartweck *et al.* (1997) have systematically combined several of the different ARL alleles in a Sanilac background, with and without phaseolin. However, they did not do this for all arcelin alleles. Another complication for their study has been the fact that ARL is tightly linked to PHA and α -AI, so when backcrossing various ARL alleles into Sanilac, some lines may have PHA α -AI whereas others may not. Goossens *et al.* (2000) have shown that accessions with combined ARL and α -AI alleles appear most resistant to bruchids. Other researchers have also found that upon transfer into a cultivated background, bruchid resistance is not as strong as it was in the original wild accession (Kornegay *et al.*, 1993). Additional evidence that ARLs are not the sole determinant of bruchid resistance comes from feeding studies with purified ARL protein and introduction of ARL transgenes into related legume species (Goossens *et al.*, 2000) where transgenic lines containing Arl-5 did not have as strong resistance as found in wild *P. vulgaris* accession G02771.

It is not known whether related *Phaseolus* species possess ARLs. Lectin-like seed storage proteins have been identified and characterized in accessions of *P. acutifolius* and *P. lunatus* (Pratt *et al.*, 1990; Blanco-Labra *et al.*, 1996; Finardi-Filho *et al.*, 1996; Mirkov *et al.*, 1994; Lioi *et al.*, 1999; Yamada *et al.*, 2001). High levels of

bruchid resistance have been demonstrated in various accessions of *P. acutifolius* (Shade *et al.*, 1987; Goossens *et al.*, 2000) and with purified *P. acutifolius* LLPs fed in artificial seeds to *A. obtectus* (Pratt *et al.*, 1990). An unusual protease inhibitor not related to lectins found in *P. acutifolius* seed may also condition resistance to bruchids (Campos *et al.*, 2004) and may account for part of the resistance seen in some accessions.

While, individual lectins and LLPs have been cloned and expressed in other species, the introgression of the complete APA locus through classical genetic techniques from *P. acutifolius* into cultivars of *P. vulgaris* remains undocumented. *Phaseolus vulgaris* and *P. acutifolius* α -AI seed proteins have been transferred into pea (*Pisum sativum*), cowpea (*Vigna unguiculata*) and Adzuki bean (*V. angularis*) for seed weevil resistance (Morton *et al.*, 2000; Yamada *et al.*, 2005). A *P. vulgaris* ARL variant was transferred into *P. acutifolius* (Zambre *et al.*, 2005).

Because *P. acutifolius* is in the tertiary gene pool of *P. vulgaris* (Singh, 2001), significant biological barriers must be overcome in order to introgress genes into common bean. The transfer to *P. vulgaris* requires careful selection of compatible parents and embryo rescue of 20 day old embryos is required to obtain viable F₁ progeny (Mok *et al.*, 1978; Federici and Waines, 1989; Jung *et al.*, 1992). The resulting plants show sterility and meiotic abnormalities that restrict further cross or self-pollination and subsequent seed set (Rabakoarihanta *et al.*, 1980). Abnormal chromosome recombination inhibits gene transfer (Haghighi *et al.*, 1988; Mejia-Jiménez *et al.*, 1994) and can interfere with transfer of quantitatively inherited traits (Anderson *et al.*, 1996; Muñoz *et al.*, 2004). In addition to choosing suitable parental genotypes, the direction of the cross is critical (Mok *et al.*, 1978; Pratt, 1983; Mejia-Jiménez *et al.*, 1994; Anderson *et al.*, 1996; Robleto and Ascher, 1996). While, some researchers have used simple backcrossing techniques, the congruity backcross method (Anderson *et al.*, 1996) and double congruity backcross (DCBC) (Mejia-Jiménez *et al.*, 2002) can increase success in producing viable fertile hybrids and facilitating recombination. While, successful interspecific hybridization between *P. vulgaris* and *P. acutifolius* has been accomplished, to date, only common bacterial blight resistance has been transferred (Singh and Muñoz, 1999).

The wild tepary bean accession G40199 was identified as highly resistant to *A. obtectus* and *Z. subfasciatus* (Mejia-Jiménez *et al.*, 2002). Resistance was introgressed into common bean from *P. acutifolius* using the DCBC method. Interspecific hybrid lines demonstrating strong resistance to *A. obtectus* were developed but resulting seeds could not germinate.

Although, the DCBC method facilitated interspecies recombination, fertility in the progeny was not restored.

The mechanism of bruchid resistance in G40199 accession remains to be discovered. If LLPs condition resistance in G40199, introgression of these polypeptides into common bean cultivars through interspecific hybridization followed by one or two backcrosses should be sufficient to restore plant fertility. A simple backcross procedure should be sufficient to transfer the APA locus, but other introgression methods will be required if resistance is more complex. The objective of this study was to generate *P. vulgaris* interspecific hybrid lines with introgression of APA locus from G40199 to determine its role in conditioning bruchid resistance.

MATERIALS AND METHODS

Interspecific hybridization and plant maintenance: Two *P. acutifolius* accessions were used in this study. G40199, a wild accession resistant to bruchids, was obtained from CIAT (Centro Internacional de Agricultura Tropical) Cali, Colombia. A cultivated brown seeded tepary accession (designated Brown Tepary) is maintained in the Vegetable Breeding and Genetics germplasm collection at Oregon State University, USA, where this study was carried out. These accessions were used for intraspecific genetic studies of protein inheritance and bruchid resistance in *P. acutifolius*. Of the *P. vulgaris* lines used in this study, ICA Pijao (CIAT accession No. G5773) is a Mesoamerican small-seeded black bean with upright type II growth habit and excellent intra- and interspecific combining ability. Rojo, an elite Andean cultivar with large red seed was obtained from Dr. Susan Nchimbi-Msolla at the Sokoine University of Agriculture breeding program (Morogoro, Tanzania). 5-593 (USDA National Plant Germplasm System accession No. PI 608674) is a small-seeded Mesoamerican black bean developed by Mark Bassett at University of Florida as a common background for genetic stocks. Both Rojo and 5-593 have determinate type I growth habit and are early maturing.

Interspecific hybrids were obtained by rescue of F_1 embryos generated from crosses between G40199 used as the pollen parent and ICA Pijao, Rojo or 5-593. A limited number of interspecific crosses were made between ICA Pijao and F_2 from the cross G40199×Brown Tepary (BTF_2) to produce a three way cross involving the two tepary bean genotypes.

Embryos were excised from immature pods 22-28 days after pollination. Pods containing immature embryos were surface sterilized in a 70% commercial bleach solution for 10 min followed by a 5 min suspension in 70% ethanol and three rinses in sterile distilled water. The immature testa

was removed and embryos were grown *in vitro* on a semi-solid MS-culture medium as described by Mejia-Jiménez *et al.* (1994) with addition of glutamine (200 mg L^{-1}), casein hydrolysate (200 mg L^{-1}) and myo-inositol (100 mg L^{-1}). Following embryo germination and preliminary growth, plantlets were transferred into potting soil and acclimatized at high relative humidity. Plants were grown under natural light at 26-27°C. Because all F_1 plants were highly sterile, they were backcrossed as females to ICA Pijao. BC_1F_1 plants were grown and backcrossed again to ICA Pijao to produce BC_2F_1 seeds. At the same time, congruency backcrossing was initiated by crossing some BC_1F_1 lines to G40199.

Analysis of major seed storage proteins: Seed storage proteins were extracted from mature dry seeds by grinding cotyledons of individual seeds to obtain a fine powder; alternatively, where hybrid seeds needed to be saved for further planting, the end of the seed distal from the embryo were rubbed on sand paper to obtain fine powder. The flour (0.5 g) was dissolved in 300 μL of extraction solution (0.5 M NaCl, 0.25 M ascorbic acid pH 2.4) and homogenized by gentle shaking and occasional vortexing for 30 min. The mixture was left to settle at room temperature for 30 min then centrifuged at 20,000 $\times g$ for 10 min. For SDS-PAGE protein separation, 10 μL of supernatant was mixed with equal volume of cracking buffer (0.625 M Tris-HCl pH 6.8, 2 mM EDTA, 2% SDS, 1% 2 β -mercaptoethanol, 0.05% Bromophenol blue) and was heated for 5 min at 95°C. The denatured polypeptides were size-separated by electrophoresis on 12% SDS-PAGE gel (BIORAD) followed by staining with 0.1% Coomassie brilliant blue R-250 in 40% methanol and 10% acetic acid solution and destained in 40% methanol and 9% acetic acid. Polymorphic electrophoretic protein profiles of different sizes were scored with reference to the 33 kDa protein subunit from G40199 and electrophoretic mobility of size standard proteins. Polypeptides corresponding to 33 kDa protein from *P. acutifolius* were scored as present or absent for each individual seed in a given line. Segregation of the LLPs was analyzed in 11 F_3 seeds from each of 116 F_3 families by Chi-square test for goodness of fit to Mendelian ratio from the Brown Tepary \times G40199 cross. LLP profiles similar to those observed in the *P. acutifolius* intraspecific cross was scored among interspecific F_1 hybrids and in the backcross generations obtained from the crosses between ICA Pijao \times G40199 crosses. Seed proteins from the rescued F_1 embryos were obtained using the same extraction procedures as for dry mature seed, except that a piece of immature cotyledon was collected before abortion and prior to embryo rescue.

Table 1: Oligonucleotide sequences specific for genomic DNA sequences of four lectin-like genes in tepary beans

Target genes	Primer sequence	Expected size (bp)	Accession No.	References
Arcelin-like (ARL2)	Forward: 5'GCT TCC TCC AAC TTA CTC TCT AG 3' Reverse: 5'ATG TGG TGT GAT CGG GGA ACT CG 3'	800	AF255724	NCBF -direct submission
Tepary bean	Forward: 5'GCT TCC TCC AAG TTA CTC TCC CT 3' Reverse: 5'CCT TCA GAT TTT TGG TCC TTA AC 3,	800	U10350	Mirkov <i>et al.</i> (1994)
Arcelin (ARC) α -AI	Forward: 5'CTT CCT CCA AGT TCT GCA GTG TG 3' Reverse: 5'ATG TGG TGT GTT GGG AGA ACT TA 3	750	AB062420	Yamada <i>et al.</i> (2001)
PHA	Forward: 5'CTT CCT CCA ACT TCT CCA CTG TC 3' Reverse: 5'CGA AGT TGG CGA GAT TCA AAC C 3'	830	U10416	Mirkov <i>et al.</i> (1994)

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Genomic DNA characterization of parents and hybrids:

Genomic DNA was extracted from young leaf tissue of individual plants from the F₃ tepary bean population, F₁ interspecific hybrids and backcross families of ICA-Pijao×G40199. Total genomic DNA from young leaf tissues was extracted as described by Miklas *et al.* (1993). Selected DNA nucleotide sequences for ARL, PHA and α -AI from *P. vulgaris* and *P. acutifolius* were obtained from NCBI database where sequences had previously been deposited (Mirkov *et al.*, 1994; Yamada *et al.*, 2001). Primers were designed by aligning all nucleotide sequences of the four possible genes in order to generate polymorphic gene specific oligo-nucleotides for each gene. Default settings of the Oligo-Tech analysis program (<http://www.oligoset.com>) were used for primer designing, optimization and determination of melting temperature for each forward and reverse primer (Table 1).

PCR and pertinent primers were used to specifically amplify DNA fragments from total genomic DNA extracted from leaf tissues. PCR conditions were optimized to attain stringent conditions for specific amplification of a single DNA size fragment corresponding to an approximate size of each corresponding gene. The optimum PCR conditions consisted of 5 min initial denaturation of template DNA at 94°C followed by 35 cycles of 94°C for 30 sec, 62°C for 40 sec and 72°C for 60 sec followed by a 5 min final extension at 72°C. A PCR reaction volume of 20 μ L contained 15 ng of genomic DNA, 50 mM MgCl₂, 2 mM of each dNTPs, 10 μ M forward and reverse primers and 0.5 unit of Taq-Polymerase (PROMEGA). PCR amplified DNA products were separated by electrophoresis on 2% agarose gel and visualized after staining with ethidium bromide.

Genomic DNA sequencing: In order to identify nucleotide sequences from PCR products, single bands of PCR amplified DNA fragments generated by each of the ARL, PHA and α -AI primers were excised from the agarose gel and recovered into Agarose purification column using QIAQUICK gel extraction kit (QIAGEN®). Purified PCR products were used for genomic DNA sequencing at the Centre for Gene Research and Biotechnology at Oregon

State University using an ABI 3730 capillary sequence machine. Gene specific nucleotide sequences were generated in separate reactions using the forward and reverse primers. Genomic DNA sequences from parents and interspecific hybrids were compared and subjected to a BLAST search for sequence difference or identity by alignment with database for lectin-like DNA sequences. Resulting genomic DNA sequence was translated into amino acid sequence where consensus amino acid sequence reading frame(s) generating high sequence identity to legume LLPs were used to identify the corresponding protein in the NCBI database and sequences were aligned by Clustal-W program to determine sequence identity with other related lectin proteins.

RESULTS

Inheritance of lectin-like proteins in the *P. acutifolius*

background: Total seed storage protein profiles from mature seeds of G40199 and Brown Tepary were visualized by SDS-PAGE to identify polymorphic polypeptide bands. One major polymorphic band of approximately 33 kDa was present in G40199 but not in Brown Tepary (Fig. 1).

This polymorphic protein complex also segregated in an F₂ population of G40199 × Brown Tepary and in the F_{2,3}, 30 families were homozygous for the presence of this polypeptide band. Sixty-four families segregated and 22 families were homozygous for the absence of this seed polypeptide. The polymorphic fragment segregated in a 1:2:1 as expected for a single dominant allele ($\chi^2 = 3.47$, $p = 0.18$).

Interspecific hybridization: A total of 127 F₁ hybrid plants from the ICA Pijao × G40199 cross were recovered from embryo culture. Of these, 33 developed into mature F₁ flowering plants. Eighty-four F₁ plants were crippled and chlorotic and died after they were transferred into soil culture or during acclimatization (Table 2). Similar numbers of embryos were rescued from crosses to Rojo and 5-593, but their survival when transferred to the greenhouse was much lower (Table 2).

Table 2: Interspecific hybridization and embryo rescue efficiency for three *P. vulgaris* parents (ICA Pijao, Rojo and 5-593) crossed to two *P. acutifolius* parents (G40199 and BTF₂)

Variables	Cross combination			
	ICA Pijao × G40199	Rojo × G40199	5-593 × G40199	ICA Pijao × BTF ₂
Embryos developing int. plantlets	127	143	118	8
Plants surviving in soil	56	12	36	4
F ₁ s flowering and used in backcrosses	33	0	4	4
BC ₁ F ₁ seed set on F ₁ plants	43	0	1	4

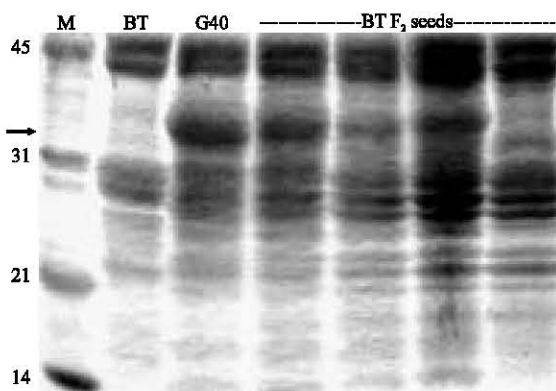


Fig. 1: Total seed storage protein from mature seeds of Brown Tepary (BT), G40199 (G40) and F₂ intraspecific hybrids (BT F₂ seeds). Prestained protein standards are shown in column labeled M and the 33 kDa protein fragment position is marked by the arrow

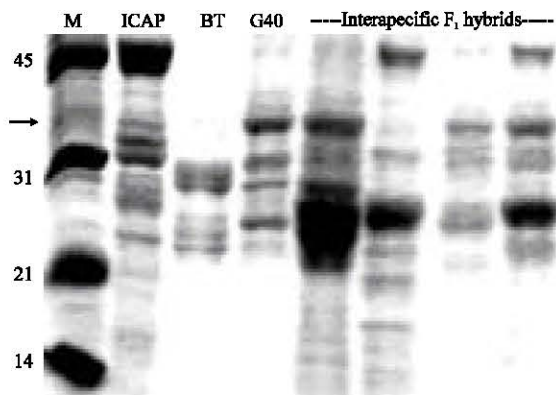


Fig. 2: Seed storage proteins profiles from F₁ interspecific hybrids (ICA Pijao×G40199 cross). Lanes M, Prestained protein standard; ICA Pijao (ICAP); Brown Tepary (BT); G40199 (G40) and interspecific F₁ hybrids. Twenty two to 28 days old cotyledon tissue was used for interspecific hybrids, mature seed for parents. Arrow indicates the position of a ~33kDa protein fragment

None of the Rojo × G40199 crosses flowered because most of the plants were chlorotic and crippled. Crosses of

5-593 × G40199 produced four dwarf plants that flowered and were backcrossed to ICA Pijao. They were not backcrossed to 5-593 because they were difficult to synchronize in flowering time with the recurrent parent. Interspecific F₁ hybrid plants were highly male sterile and mostly female sterile. They were used as females in backcrosses to ICA Pijao and 43 BC₁F₁ plants were generated. Fertility was improved in the second generation of backcrossing to ICA Pijao as demonstrated by the increased number of seeds per pod and reduced number of parthenocarpic pods (data not shown) and self fertility was nearly normal.

Characterization of LLPs among hybrids: Interspecific hybrids were evaluated for the presence of a 33 kDa polypeptide band comparable to those observed in G40199 and intraspecific tepary hybrids. It was difficult to obtain sufficient quantities of tissue from the very small cotyledons of 22-28 day embryos taken just prior to embryo rescue for protein determination. A few embryos could be excised and early expression of the 33 kDa polypeptide was observed in some of the interspecific F₁ hybrids (Fig. 2).

The novel polypeptide was similar in size to that observed in G40199. Seeds from BC₁F₁ and BC₂F₁ interspecific hybrids were also analyzed for stable introgression and expression of the 33 kDa polypeptide (Fig. 3).

We strongly suspected that 33 kDa polypeptide band was associated with a LLP and possibly arcelins, warranting characterization at the DNA level. Using four gene specific primers for arcelin (ARC and ARL-2), α amylase inhibitor (α-AI) and phytohaemagglutinin (PHA) from *P. acutifolius*, we demonstrated the presence of ARL-2, α-AI and PHA in genomic DNA of G40199 and ARC and α-AI in BT (Fig. 4).

Genomic DNA from intraspecific and interspecific hybrid plants that possessed the 33 kDa polypeptide band was subjected to PCR amplification using gene specific primers for ARL-2, α-AI and PHA. A DNA fragment of approximately 790 bp was amplified by ARL-2 primers corresponding to an arcelin-like gene in *P. acutifolius* (AF255724, NCBI database). A similar size fragment was produced by the same primers in F₁

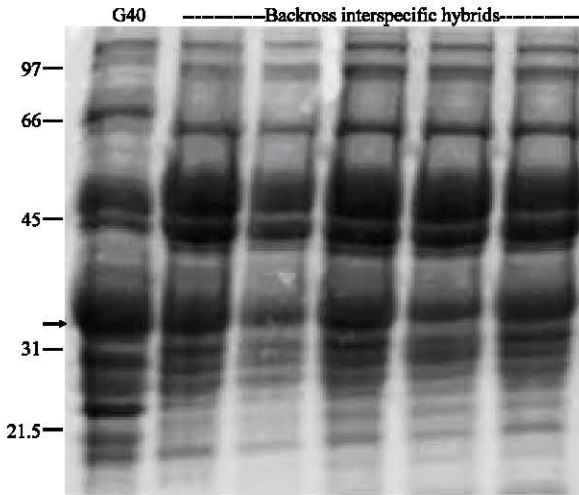


Fig. 3: Seed storage protein profiles from BC₁F₁ progenies of interspecific hybrids between G40199 (G40) and ICA Pijao . Backcross interspecific hybrids show stable integration of the ~33 kDa polypeptide band (arrow). SDS-PAGE standard kDa weights are indicated on the right

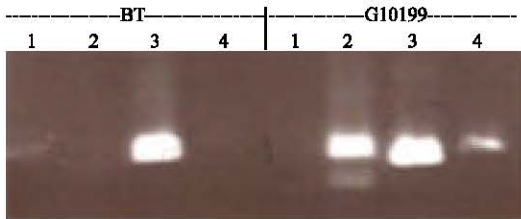


Fig. 4: Amplification of ARC, ARL-2, α -AI and PHA (lanes 1- 4, respectively) from genomic DNA of Brown Tepary (BT) and G40199 *P. acutifolius* accessions

interspecific hybrids, while no PCR products were generated by these primers in Brown Tepary or ICA Pijao (Fig. 5).

In addition, fragments for α -AI and PHA showed amplification in G40199 and BT but not ICA Pijao (data not shown). All interspecific hybrids containing ARL-2 also showed amplification of α -AI and PHA, demonstrating the transfer of the tightly linked complex locus from G40199 (Fig. 6). The DNA fragments for each gene had different molecular size with ARL-2 at 790 bp, α -AI at 750 bp and PHA at ~890 bp (Fig. 6).

On the other hand, intraspecific hybrid plants from the Brown Tepary \times G40199 F₃ population had either ARL-2 or ARC bands but did not segregate for α -AI and PHA. While, the two DNA fragments were found in heterozygous individuals, in 60 F₃ families that were

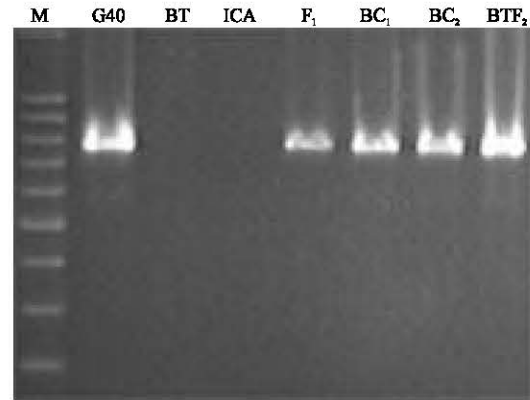


Fig. 5: Amplification of ARL-2 from genomic DNA of G40199 (G40), Brown Tepary (BT), ICA Pijao (ICA) and derived interspecific hybrids (BC₁F₁: BC₁, BC₂F₁: BC₂, BT \times G40199 F₂). Lane M is a 100 bp DNA standard

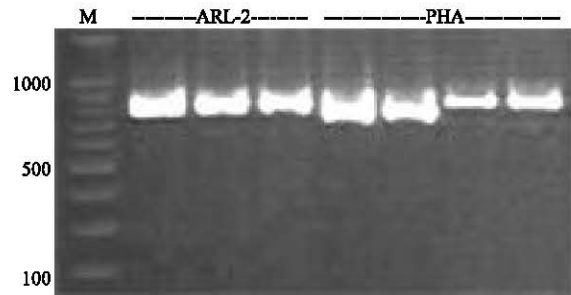


Fig. 6: PCR amplification of ARL-2, α -AI and PHA from genomic DNA of G40199 (lanes 2, 5, 7), F₁ interspecific hybrid (lane 3) and BC₁F₁ (lanes 4, 6, 8). Lane 1: M is a 100 bp DNA standard



Fig. 7: Allelic relationship of ARL-2 and ARC and inheritance pattern for α -AI. Lane 1: G40199; Lane 2: BT; Lane 3: ICA Pijao, Lanes: 4-6, BC₂F₂ interspecific hybrids and lane 7, BT \times G40199 F₃ individual that lacked the 33 kDa protein

homozygous (based on SDS-PAGE gel of 11 plants per F₃ family), the two DNA fragments were never amplified simultaneously from genomic DNA (Fig. 7) suggesting that the two arcelin-like variants may be alleles at the same locus, or in tightly linked loci. In addition, DNA fragments generated by ARC primers were never found in genomic DNA of interspecific hybrids derived solely from G40199.

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gDNA-ARL2 3 -ASSEKLLERA LEWESAFYSD EQFENTXTNF DFEKQSDADT ERLLIQRDAT ISEGGRLRLR 60
ARL2-pa MASSKLLERA LFLLLPTRAI E-ADDTXTNF DFEKQSDADT ERLLIQRDAT ISEGGRLRLR
***** * : * : ***** ***** ***** *****
gDNA-ARL2 61 SVSSEEDRWV DSMERAFYSD FIIQIRDSTGN LESTHINFSF IIRAFNGGNS AYGLAFSLVF 120
ARL2-pa SVSSEEDRWV DSMERAFYSD FIIQIRDSTGN LESTHINFSF IIRAFNGGNS AYGLAFSLVF
***** * ***** * ***** * ***** * ***** *
gDNA-ARL2 121 VGSQPKGKRE YLSLRFDAHA VVAFAFNILNN SVIDIVYSYS RSHTEGVDEN KNGGKTIQVQ 180
ARL2-pa VGSQPKGKRE NLHLRFDAHA VAV-FNEVSN VHESTSTPR LAQRREFAIST NNGGKTIQVQ
***** ***** * * * * * * * * * * * * * * * * * * * * * * * * * * * *
gDNA-ARL2 181 ITYESPWKNE RYVLFHFKSN VQYEVDFDAP L-LENCVDNS VQRWVGFQAT -GLKSTIAS 240
ARL2-pa ITYESPKKRL KTVLPSTHNS VQ--YDFKAP LYLEHEVYER VE--VEFQAT SGLTEETIET
***** * : * * * * * * * * * * * * * * * * * * * * * * * * * * * *
gDNA-ARL2 241 HDILSWEFSS E----- 270
ARL2-pa HDILSWEFSS EPPDHTISEP SHILLNHL
***** *
    
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Fig. 8: Deduced amino acid sequence derived from genomic DNA sequence of ARL-2 from G40199 (gDNA-ARL2) aligned with amino acid sequence from ARL-2 from an unidentified *P. acutifolius* accession deposited in NCBI database

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α-AI-pa 1 MASSKFCQSVL SLVLFVLVLT MANSACHTSF NERSFWETHL MLQGGQATVSS 50
gDNA-αAI-G40 -SSSEAVSLP SPLPCLVLET HANSASDT-F NRESFWETHL TLQGDATVSS
(* * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
α-AI-pa 51 NGHLQLHTED SMCSAFYSAP IQIRDSTTGN VASFDTHFTI NMTSYGKANS 100
gDNA-αAI-G40 NGHLQLHTED SMCSAFYSAP IQIRDSTTGN VASTHTNFTI NITTYRQANS
*****;*** ***** ***** ***** ***** * : * * * * * * * * * * * * * * * *
α-AI-pa 101 AVGLDFALVP VQPESEGRLL GLFETPDYDR MAGNVTVEFD TFERRISIDG 150
gDNA-αAI-G40 AVGLDFALVP VQPESEGRLL GLFETPDYDR MAGNVTVEFD TLERRISIDG
***** ***** ***** ***** * * * * * * * * * * * * * * * * * * * * * *
α-AI-pa 151 NYNDIESVPW NVDDYDGGQA EVRITNSST KVLAVSLNL STGKSNVSA 200
gDNA-αAI-G40 NYNDIESVPW NVDDYDGGQA EVRITNSST KVLAVSLNLF STGKSNVSA
* : * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
α-AI-pa 201 RMELEKRLDD WYVQVFIGTS GVHQVAFETR DVFWSVFSSE FSQHTTSERS 250
gDNA-αAI-G40 RMELEKRLDD WYVQVFIGTS GVHQVAFETR DVFWSVFSSE FSQHTTSERS
***** ***** *****;***** * * * * * * * * * * * * * * * * * * * * * *
α-AI-pa 251 HILINQIL 258
    
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Fig. 9: Amino acid sequence alignment from translated α-AI genomic DNA of G40199 with a complete amino acid sequence for α-AI-1. (*) Identical residues, (:) Similar residues and (-) Indicate gaps

In interspecific hybrids obtained from three-way cross ICA Pijao × (BT × G40199 F₂), we did observe segregation for both of the ARC and ARL-2 fragments, suggesting that the BT genotype contain the ARC gene in that were introgressed into interspecific crosses that carried both alleles.

Genomic DNA sequencing: Genomic DNA sequences were obtained from PCR amplified products of polymorphic DNA fragments corresponding to ARL-2, ARC and α-AI and were compared to published sequences for the genes originally used to design primers. DNA sequences for ARL-2 from G40199 and derived interspecific hybrids showed a 94% identity to the published ARL-2 sequence, with a 70% amino acid sequence identity following sequence alignment by Clustal-W (Fig. 8).

Similarly, genomic DNA from the sequenced fragment generated by α-AI primers in G40199 exhibited 95% nucleotide sequence identity to α-AI described in tepary bean by Yamada *et al.* (2001). Amino acid sequence from G40199 demonstrated 87% identity (Fig. 9) to α-AI-1 and 80% sequence identity to α-AI-2 of tepary bean (Yamada *et al.* 2005). The presence of α-AI sequence in some interspecific hybrid lines confirms successful transfer of part of the complex locus encoding the lectin-like gene family from tepary bean to common bean cultivar ICA Pijao.

DISCUSSION

The inheritance of LLP genes from tepary bean demonstrated simple Mendelian inheritance in a segregating population of intraspecific hybrids between

wild tepary G40199 and cultivated Brown Tepary. This intraspecific cross provided us with essential genetic information for the inheritance of the 33 kDa seed storage protein profile, which facilitated interspecific hybridization and selection of hybrids.

The success of interspecific hybridization between *P. acutifolius* and *P. vulgaris* was highly dependent on the cultivars of common bean selected for hybridization. ICA Pijao was most amenable for generating viable interspecific hybrids via., embryo rescue and simple backcrossing. Rojo was used as a parent because it is an elite cultivar released in Tanzania that has desirable quality attributes and is regionally adapted and direct transfer of the trait rather than using a bridge parent would be desirable. However, if we had relied solely on Rojo to facilitate interspecific transfer, then the project would have failed. Five to 593 was used in crossing attempts because it is a widely used genetic stock. It is early maturing with compact determinate habit and has previously demonstrated compatibility in interspecific hybridization with *P. coccineus* (Ferwerda and Bassett, 2000). Progeny were obtained from crosses with 5-593, but these had more abnormalities than did progeny from ICA Pijao crosses. Given the obvious superiority of ICA Pijao in generating F₁ progeny, the cultivar was then used to produce the BC₁ and BC₂ generations.

The 33 kDa polymorphic band identified in G40199 and transferred into *P. vulgaris* corresponds to the molecular size of ARL and AL proteins (Pratt *et al.*, 1990; Dillen *et al.*, 1997). Similar-sized seed proteins associated with ARL, α -AI and PHA have been characterized in accessions of *P. acutifolius* (Yamada *et al.*, 2001, 2005). The presence of LLPs in a bruchid-resistant tepary bean accession and their segregation among intraspecific and interspecific hybrids may be among the factors linked to high antibiosis activity to bruchids.

When genomic DNA derived sequences were aligned with nucleotide sequences of genes from the NCBI database, G40199 showed 94% DNA and 72% amino acid sequence identity to ARL-2. The accession that was used to produce the original ARL-2 sequence is unknown, but was thought to be a cultivated tepary variety bought from a commercial market (Chrispeels, personal communication). It is also not known whether the original ARL-2 seed storage protein is a functional protein that conditions resistance to bruchids, since no evidence of insect feeding trials have been reported. As such, researchers who deposited the sequence designated it as arcelin-like. We believe that the ARL allele in G40199 is functional based on feeding trials with *A. obtectus*.

A second gene amplified by primers designed for α -AI produced a relatively similar fragment to α -AI-1 described by Yamada *et al.* (2005). α -AI proteins inhibit α -amylase activity of *Z. subfasciatus* and *C. maculatus* in peas but have not been tested and there is no prior evidence of alpha amylase inhibitor alone to confer resistance against *A. obtectus*. Even so, the successful transfer of this protein into common bean in combination with arcelin variants should contribute to enhanced bruchid resistance if a synergetic mechanism is involved. Genomic DNA from PHA fragment was not sequenced but its presence as part of the APA locus was demonstrated in the parent G40199, Brown Tepary and interspecific hybrids.

Although, strategies have been developed in the past to breed for resistance to bruchids by normal backcrossing of lectin-like genes from wild common bean, little success has been made to transfer specific arcelin, phytohaemagglutinin and α -AI genes from *P. acutifolius* to cultivars of *P. vulgaris*. Among wild accessions of common bean, G02771 contains Arl-5 and has a complete set of LLPs at the APA locus (Paes *et al.*, 2000; Goossens *et al.*, 2000; Kami *et al.*, 2006). G02771 demonstrated high level of resistance to *Z. subfasciatus* and moderate resistance to *A. obtectus*, a resistance that was associated with factors linked to Arl-5. Unfortunately, Goossens *et al.* (2000) did not find similar level of resistance to bruchids on transgenic beans expressing arcelin 5 indicating the importance of the entire APA locus for high resistance. We suggest that the high levels of resistance to both species of bruchids found in G40199 may be due to the simultaneous presence and interaction of the three APA genes. G40199 may have additional factors that amplify transcription of the APA locus genes thereby increasing levels of insecticidal proteins relative to other seed storage proteins. A similar mechanism may be associated with antibiosis to bruchids in the other tepary bean accession used in this study. Brown Tepary also had a complete APA locus, although a different ARL variant was observed. It also demonstrated partial resistance to *A. obtectus* (unpublished data). The difference in arcelin variants between wild and cultivated accessions of *P. acutifolius* may contribute to variable levels of resistance to bean weevils. Preliminary studies on these intraspecific tepary genotypes indicated promising levels of resistance to *Z. subfasciatus* and *A. obtectus*.

Because the genes encoded in the APA locus are thought to be free of introns (Kami *et al.*, 2006) we expect that the genomic DNA sequences observed hereto will be translated into mature active protein products. Further detailed characterization of transcriptional and

translational levels of the identified lectins and lectin-related genes from tepary beans are being conducted in conjunction with characterization of the same proteins among interspecific hybrids. Any changes in protein stability or modification of the proteins among hybrids at different backcross generations of interspecific hybrids remain to be confirmed. Meanwhile, if mRNA translates into stable functional proteins of the three candidate-APA protein variants among interspecific hybrids, this will provide conclusive evidence that functional proteins have been transferred. Proteomic characterization and identification of other associated proteins that reside in the same fragment may provide more information on the components of the 33 kDa protein and associated subunits that are actually expressed in the seeds of the wild accession G40199. Parallel to gene-protein expression studies, characterization of interspecific hybrids to determine if the introgressed tepary seed proteins co-segregate with bruchid resistance needs to be determined in bruchid feeding trials.

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