AN ABSTRACT OF THE DISSERTATION OF

Paul Mbogo Kusolwa for the degree of Doctor of Philosophy in Horticulture

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Title: Breeding for Bruchid Resistance in Common Bean (*Phaseolus vulgaris* L.): Interspecific Introgression of Lectin-like Seed Proteins from Tepary Bean (*P. acutifolius* A. Gray), Genetic Control and Bruchid Resistance Characterization.

Abstract approved:_____
James R. Myers

Interspecific hybridization was initiated between wild *P. acutifolius* accession G40199 and *P. vulgaris* to introgress resistance to *Acanthoscelides obtectus* (bean seed weevil). F_1 interspecific hybrids were recovered by embryo rescue and maintained until flowering. Mostly sterile hybrids were backcrossed twice to common bean cultivar ICA Pijao to recover sufficient fertility for the lines to reproduce without assistance. The inheritance of a 33 kDa seed storage protein from accession G40199 was studied in an F_2 intraspecific population from a cross of G40199 and a cultivated Brown Tepary accession. G40199 possessed the protein but Brown Tepary did not. The protein was inherited as a single dominant gene in a F_2 population of 116 individuals. Following these observations, interspecific hybrids were progeny tested for the introgression of a 33 kDa protein from accession G40199. Backcross interspecific introgression lines were used as bridge parents to transfer the 33 kDa protein into the large red seeded cultivar Rojo adapted to Tanzania, and into a phaseolin null backcross breeding line of Rojo with the objective of introgressing resistance to *A. obtectus* into a Sub-Saharan Africa variety. Genomic, proteomic and phylogenetic characterization of genes associated with the 33 kDa proteins were

conducted in order to identify the mechanism of resistance found in accession G40199. Genomic DNA encoding a family of lectin-like seed storage proteins (the complex APA locus) was amplified by PCR using primers for arcelin, phytohaemagglutinin and alpha amylase inhibitors. The PCR products were used as molecular markers and co-segregated with the 33 kDa protein. Analyses of mRNA expression identified two arcelin variants, ARL-3^{pa} and ARL-4^{pa}, as expressed in G40199 and its derived interspecific hybrids. MS-MS proteomic analysis of seed protein profiles in G40199 demonstrated the presence of protein peptides with amino acid sequences corresponding to the two arcelin variants and phytohaemagglutinin (PHA) protein subunits. Essentially similar protein peptides were observed in the introgression lines. Phylogenetic analysis demonstrated the difference of the two arcelin variants in G40199 that clustered with *P. acutifolius* arcelin-like proteins and were separated from those of *P. vulgaris*. Seeds from G40199 and BC_2F_3 interspecific hybrid lines were subjected to an *A. obtectus* feeding trial at OSU. Introgression lines expressing the homozygous 33 kDa protein demonstrated significant bruchid resistance. This resistance was exhibited as delayed insect emergence with a mean of 63 days for 50% F_1 adults after inoculation compared to 44 days to 50% adult for ICA Pijao. In addition, reduced size and weight of adults, and reduced number of adults was observed in a period of 72 days after bruchid infestation. G40199 was completely resistant with no emerging adults. The 33 kDa protein which is linked to co-expression of ARL-3^{pa}, ARL-4^{pa} and PHA protein subunits is a contributing factor to the observed resistance to *A. obtectus* among interspecific hybrids.

Breeding for Bruchid Resistance in Common Bean (*Phaseolus vulgaris* L.): Interspecific Introgression of Lectin-like Seed Proteins from Tepary Bean (*P. acutifolius* A. Gray), Genetic Control and Bruchid Resistance Characterization.

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Paul Mbogo Kusolwa, Author

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CONTRIBUTION OF AUTHORS

Dr. James Myers contributed in reviewing the manuscripts and provided general advisory guidance in the conduct of the researches. Joel Davis perormed the initial analysis that identified the 33kDa protein in G40199 and and provided technical assistance in laboratory analysis for proteins and molecular analysis during genomic DNA characterization.

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DEDICATION

To my Parents and my beloved family.

Breeding for Bruchid Resistance in Common Bean (*Phaseolus vulgaris* L.): Interspecific Introgression of Lectin-like Seed Proteins from Tepary Bean (*P. acutifolius* A. Gray), Genetic Control and Bruchid Resistance Characterization.

CHAPTER 1

Introduction

ECONOMIC IMPORTANCE OF COMMON BEAN

Common bean (*Phaseolus vulgaris* L.) is among the members of edible legumes that belong to the family Leguminosae/Fabaceae, subfamily Papilionideae described in the tribe Phaseoleae, sub tribe Phaseolinae in the section Phaseoli (Debouck 1991). Cultivars and landraces of *P. vulgaris* are derived from independent domestication of wild common beans in the Andean and Middle American centers (Chacon et al. 2005). The process of domestication and selection produced two major classes of edible bean; dry beans and green or snap beans. Today, fresh green pods with immature seeds -green beans, fresh shelling beans and dry pulse beans (dry beans) are widely consumed. Dry beans dominate total bean production.

Common bean is the third most important food legume crop of the world after soybean and groundnuts; it provides an important source of dietary proteins, carbohydrates, minerals and fiber essential in human nutrition worldwide. A high per capita consumption of 13 to 40 kg yr^{-1} of dry bean is observed in developing countries, especially within low-income families in urban and rural areas (Singh 1999). Dry bean remains the primary source of dietary protein in most of the developing countries where it

is regarded as poor man's meat. In addition to its central nutritional and dietary role, dry bean is gaining importance as a source of household income for small scale farmers, who sell a portion of the produce through middlemen to remote urban dwellers and exporters.

Common bean is grown worldwide where temperatures are moderate and some water is available. Among developing countries, Eastern and Southern Africa, parts of Asia, Central and South America, are the largest producers and consumers throughout the year. It is estimated that over 14 million hectares of the world's arable land is dedicated to common bean production, which amounts to more than 11 million tons worldwide (Singh 1999; FAO 2005). Any effort to alleviate constraints affecting the production, processing or marketing of common bean that will result in increased productivity and sustainability of the commodity in the region will potentially contribute to improved health and living standards, and to the national economy.

CONSTRAINTS AND ECONOMIC IMPORTANCE OF BEAN BRUCHIDS

Common bean production in the tropical and subtropical countries is constrained by many biotic and abiotic factors affecting crop productivity and crop quality during the growing season. Reduced productivity is associated with low yield per unit area, limited number of adapted varieties, poor or unreliable seed quality, and various diseases and pests that affect the bean crop. Post-harvest losses may reduce market value after the crop is in storage.

Dry bean seeds being rich in proteins, carbohydrates, and lipids are subject to predation by post-harvest pests including bean weevils (bruchids). Bruchids attack dry bean seeds both in the field before harvest and in storage warehouses. The adult weevils

deposit eggs on the surface of bean pods or directly on the seeds, and the eggs hatch into larvae that burrow through the pods and into the seeds to feed on the nutritious cotyledons. The larvae remain inside the seed during metamorphosis from larva to adult, when they emerge to continue the reproduction cycle even while beans are in storage. Over the course of the storage cycle, quantity and quality losses accumulate with successive generations of the pest.

Damage is directly related to the number of larvae that hatch and burrow into the seeds. Each emerging adult leaves a serious perforation, with resultant weight loss of the seed. Adult bruchids cause no direct damage to the beans in storage, but females can lay 30 to 60 eggs that perpetuate the cycle depending on the species and host (Parsons and Credland, 2003). Though reports of economic damage vary, weevil damage in the warehouses can result in as much as a 48 % reduction in quality and quantity (Slumpa & Ampofo, 1991). A 5-20 % seed-weight loss has been reported, and bean seeds can sometimes be turned into hollow shells filled with powdered cotyledon and insect frass (Schoonhoven et al. 1983, Schoonhoven & Cardona, 1986). The extent of crop loss may vary depending on location, the species of weevil, storage conditions, and period of storage. The longer that beans are kept in warehouses, the greater the chance of complete crop loss. The average worldwide loss caused by weevil damage ranges from 7-40% of marketable beans. Other important losses include nutritional quality loss due to protein and carbohydrate degradation, low market value of edible bean seeds and eventually loss of seed viability.

To avoid losses, farmers limit the time that they keep their harvest. Many farmers sell the crop (usually at low price) shortly after harvest. Another production strategy that

farmers use is to not produce a large quantity during any one season for the fear of such post-harvest losses. Where bean weevils are prevalent, significant market price fluctuations are observed throughout the year, leading to short periods of high quantity and long periods of low quantity and/or quality. The period of short supply can vary from three to six months in regions with bimodal rainfall, and six to eight months where unimodal rainfall is the rule. Small scale farmers who produce only enough food to subsist from one growing season to the next are the most vulnerable to environmental threats, contributing to low household income and food insecurity. Lack of bruchid resistant cultivars, poor storage facilities, and inefficient control strategies contributes to the low productivity that discourages farmers from engaging into intensive bean production. Cultivar improvement for bruchid resistance is among the important strategies of mitigating biotic factors affecting productivity of beans in Sub-Saharan Africa. Dry bean cultivars that delay or prevent development of the predominant bruchid species will contribute to a stable supply of dry beans in the region, especially where only a single crop is produced per year. Bruchid resistant bean cultivars should also have a stabilizing influence on market prices during the dry season.

BRUCHID SPECIES OF *PHASEOLUS* BEANS

Bruchids belong to the order coleoptera in the family bruchidae. Within this family, a subfamily bruchinae contain the most economically important bruchid species that feed on cultivated and wild bean species and specifically dry beans. Several genera are placed in this subfamily but the two main bruchid species thought to have co-evolved with *Phaseolus* beans are the common bean weevil (*Acanthoscelides obtectus* Say.) and the Mexican bean weevil (*Zabrotes subfasciatus* Boh.). In their natural state, the two

bruchid species feed on wild common, tepary (*P. acutifolius* A. Gray), runner (*P. coccineus* L.) and lima and (*P. lunatus* L.) beans.

The two species are now distributed globally in nearly every bean producing region. Infestations are rare in northern latitudes but can occur. Bruchids are most significant in warm tropical areas with a single crop per year. Although these two species can be found in the same region, their occurrence is highly influenced by weather and climate (Abate and Ampofo, 1996). Warm temperatures favor the development of *Z. subfasciatus* while the *A. obtectus* is better adapted to high altitudes with a cooler tropical climate coinciding with harvest time. While *Z. subfasciatus* only infests in storage, *A. obtectus* initiates infestation in the field, which continues in storage. In Tanzania, *A. obtectus* is the most prevalent species in the bean producing regions of the country, accounting for about 73% of the bean bruchid species collected in seed lots across the country (Misangu, 1997). This is not surprising given the fact that most bean production in Tanzania takes place at mid- to high-altitudes.

A. obtectus has the greatest genetic variation, with a worldwide distribution along wide geographic ranges (Gonzalez-Rodriguez et al. 2000). Because *Z. subfasciatus* only infests bean seeds in storage by directly attaching eggs to the testa, bean seeds that are placed in bruchid-proof storage will be protected from this species. *A. obtectus* will attack maturing and dry bean pods in the field and continues laying eggs loosely on seeds in storage, making it impossible to control this pest with insect-proof storage. Based on its life cycle and climatic adaptation to bean producing areas, *A. obtectus* presents a significantly greater threat to bean production than does *Z. subfasciatus* in East Africa.

Bean cultivars grown in Tanzania are highly susceptible to *A. obtectus* (Misangu, 1997) and no resistant varieties have been produced for this pest.

Although both species are present in different production areas of Tanzania, developing genetic resistance to *A. obtectus* will significantly reduce the incidence of seed damage from the field and therefore minimize the amount of initial *A. obtectus* larvae that will be passed into storage.

CONTROL STRATEGIES AGAINST BEAN BRUCHIDS

In East and Southern Africa, subsistence farmers employ several strategies to control bean bruchids, including mixing and storing seeds with threshing residues, dust, and wood ashes. These materials pack into the interstitial spaces between seeds and restrict bruchid movement inside the storage containers. These compounds also irritate the adult bruchids and reduce egg laying potential. Frequent sun drying of bean seeds may be used because exposure of seeds to extreme warm and dry conditions is unfavorable to bruchid development. Tumbling of storage containers causes damage to eggs laid on the surface of the bean seeds and disorients the larvae from penetrating the seed (Quentin et al. 1991) but is only practical and effective for *A. obtectus* on small seed-lots of stored beans. Cold storage rooms can be used as they retard the developmental and reproductive activities of weevils where seeds are kept at cool temperatures of 4–5^{o}C. However, this technique is not feasible for low income small-scale bean producers in tropical regions due to high cost of investment and unreliable power supplies. Other control methods involve the use of vegetable oils and botanical herbs (Schoonhoven, 1978; Mazzonetto & Vendramim, 2003) that can be mixed with bean seeds before beans are stocked in the storage warehouses. These plant products

include extracts from leaves of *Eucalyptus citriodora*, neem leaves or seeds (*Azadirachta indica* L.), tobacco leaves, leaves of coriander, *Chenopodium ambrosioides*, citrus rinds, and tephrosia (*Tephrosia spp*.) which act as insecticides and red or black peppers used as irritants. Maize oil, soy oil concentrated juice from banana plants appear to suffocate various insect stages and penetrate into eggs reducing eggs' fertility thus protecting beans from *A. obtectus* and *Z. subfasciatus* (Schoonhoven and Cardona, 1986; Baier and Webster 1990; Slumpa and Ampofo 1991). Other local management strategies employ timely harvesting, and use of air-tight containers to minimize bruchid infestation before and during storage. Bean seeds may be fumigated and or dusted with insecticides immediately after harvesting. Because chemical control of bruchids is costly and requires significant infrastructure, it is mainly practiced by large scale farmers. Most of these cultural and chemical control strategies suggested by Schoonhoven and Cardona (1986) for East Africa do not provide effective control of bean bruchids from one cropping season to another unless used repeatedly. Biological control strategies have been suggested to reduce *A. obtectus* infestation in the field using the parasitoid *Horismenus ashmeadii* (Dalla Torre) (Hymenoptera -Eulophidae) that feeds on the larvae of *A. obtectus* (Schmale et al. 2002) but the parasitoid was not effective in storage.

A cost effective strategy is required in order to provide efficient and sustainable control of bean bruchids. A combination of genetic resistance, biological and cultural control methods can be effective. Most resistances to insects found in plants are quantitatively inherited, making breeding for resistance a slow and laborious process. Resistance to bruchids in common bean is unusual in that it is qualitatively inherited and generally well understood. In efforts to develop bruchid resistant beans, researchers

identified and introgressed different genetic resistance factors from wild relatives into common bean. The major resistance factors associated with seed proteins in mature bean seeds are arcelins (Osborn et al. 1888b). Arcelins are found in wild *P. vulgaris* from Mexico and do not exist in the cultivated gene pool. Arcelins are associated with the lectin or the arcelin-phytohaemagglutinin-alpha amylase inhibitor (APA) complex locus. There are possibly additional unknown factors linked to the locus as well (Fory et al. 1996; Goosens et al. 2000; Kami et al. 2006; Nishizawa et al. 2007).

Genetic resistance to *Z. subfasciatus* has been identified and incorporated into some common bean varieties (Cardona et al. 1990; Misangu 1997). While the arcelin allele *Arl-1* has been transferred into common bean cultivars in Tanzania and conditions resistance to *Z. subfasciatus*, no stable resistance to *A. obtectus* has been developed in any of the varieties of common bean. Low to moderate resistance conditioned by *Arl-2*, *Arl-4* and *Arl-5* has been reported but has not been stably transferred to commercial varieties (Kornegay & Cardona 1991; Cardona et al. 1992; Hartweck et al. 1997).

In addition to wild accessions of common bean, high levels of resistance to the two major bruchids pests have been identified in wild and cultivated accessions of tepary bean (Shade et al. 1987; Pratt et al. 1990). A highly bruchid resistant tepary bean accession G40199 was identified at CIAT and by Goosens et al. (2000). It demonstrated increased adult mortality, reduced adult emergence, and prolonged larval developmental time (Shade et al. 1987). Introgression of resistance factors from tepary bean by interspecific hybridization is expected to be a feasible strategy for enhancing resistance to cultivars and landraces of dry beans of East and Southern Africa. The resistance mechanism in G40199 has not been identified, and this needs to be done in order to

formulate a coherent strategy for interspecific transfer. A partial screen of variation of seed protein profiles from extracts of total seed proteins reported by Myers et al. (2001) identified a polymorphic 33 kDa protein observed when this genotype was crossed with another cultivated brown tepary bean genotype. It was unknown if this protein profile is associated with weevil resistance and if they can be transferred into common bean genotypes. Characterization of possible insecticidal seed proteins residing in the polymorphic protein profiles from this tepary bean and their successful transfer into cultivars of common beans is needed. Finally, once transferred into common bean, characterization of the actual resistance among interspecific progenies through bruchid feeding trials is necessary in order to establish any linkage between the proteins and bruchid resistance. Resulting resistant progenies from the interspecific hybrids will be used as bridge parents for further genetic improvement of bean cultivars in Tanzania and other areas where bruchids present a threat to bean storage.

Transfer of this resistance to adapted varieties will provide Tanzanian bean farmers with varieties that can be stored longer with less post-harvest loss. Resistance may be justifiable if beans can be kept from weevil damage for at least 60-90 days or longer after harvest as a result of resistance conditioned by the introduction of the novel seed protein from tepary bean. In the absence of resistance and any control measures, weevil damage becomes apparent within 30-38 days of harvest. With extended time of safe storage, farmers will have greater flexibility of when to sell their beans at the best market price during the year. This will stabilize bean supply and minimize seasonal price fluctuation in the market and increased seed availability for the following production

season. Development of bruchid resistant cultivars may also reduce the use of expensive pesticides used to protect large stocks of dry beans in storage.

OBJECTIVES OF THE STUDY

The main goal of this work is to develop dry bean varieties adapted to East Africa that are resistant to bruchids by deploying the resistance factors from wild tepary bean accession G40199. The specific objectives of this work include:

1. Transfer of lectin-like seed storage proteins from wild tepary beans into a cultivated brown seeded tepary bean accession to study the heritability of resistance in a segregating population.
2. Generate interspecific hybrids between *P. vulgaris* and *P. acutifolius* and characterize the heritability and expression of arcelin-phytohaemagglutinin and alpha amylase inhibitor seed proteins from wild tepary.
3. Introgress lectin-like proteins from accession G40199 into large seeded cultivars preferred in East Africa via bridge interspecific hybrid parent.
    - Introgress tepary arcelins into Rojo cultivar
    - Develop congruity backcross lines
    - Introgress tepary arcelins into a phaseolin null bean genotype
4. Conduct a genomic characterization of arcelin-like seed storage proteins in wild tepary bean and interspecific hybrids and determine their phylogenetic relationship with other *Phaseolus* bean lectins.

5 Evaluate the association of arcelin-like proteins from tepary bean to *A. obtectus* resistance among interspecific backcross hybrid lines generated from crosses between *P. vulgaris* and *P. acutifolius*.

CHAPTER 2

Literature Review

LEGUME SEED PROTEINS AS A DEFENSE MECHANISM AGAINST BRUCHIDS

Seeds of most genera in the family Fabaceae accumulate various antinutritional compounds as defense mechanisms against microbes and predation by insects, rodents and other animals. Most of these compounds are in the form of seed storage proteins, cyanogenic glycosides, and phytic acid that accumulate in the cotyledons of seeds during seed maturation. Types and variants of seed proteins of cultivated and wild legume species have been identified and characterized (Gibbs et al. 1989; Shewry et al. 1995; Shewry and Lucas, 1997; Fernández-Quintela et al. 1997; Sales et al. 2000; Campos et al. 2004).

Common bean also contains significant amounts of seed storage proteins used for embryo and seedling development, as well as for defense against seed pests. Some of the well documented and important storage proteins in common bean seeds includes: phaseolin, lectins, phytohaemagglutinins, trypsin inhibitors, and lectin-like proteins that include arcelins and α-amylase inhibitors. Phaseolin is among the most extensively studied major storage protein of the common bean (Brown et al. 1982; Gepts, 1988) and has been used to explain the evolutionary relationship of different germplasm pools within *P. vulgaris* (Gepts 1988; Kami et al. 1995). Phaseolin is an important source of essential amino acids for animal nutrition, and unlike other bean seed storage proteins, is not associated with an antibiosis effect to insect pests. In addition to phaseolin, the

second most common group of seed proteins in common bean are those loosely called lectins or phytohemaglutinins, as well as additional lectin-like proteins (Osborn et al. 1988b; Crispeels and Raikhel, 1991).

LECTINS AND LECTIN–LIKE SEED PROTEINS

Lectins or agglutinins are among the group of proteins that reversibly bind to carbohydrate molecules (Peumans and Van Damme 1995). These sugar binding proteins are found in bean seeds, with small amounts also found in roots, leaves and sometimes in seed testa (Sales et al. 2000). Biologically, they are involved in cell-cell recognition and in specific recognition of symbiotic bacteria. In addition to their role in cell recognition, lectins provide defense against plant eating-organisms by binding to digestible sugar molecules, thus interfering with carbohydrate metabolism of the animal or microbe. Lectins are highly stable at a wide pH range and are resistant to heat and to insect proteases. This stability is important to their role in protecting bean seeds from insect and animal predation.

Various species of the genus *Phaseolus* contain a family of related lectins and lectin-like proteins (LLP) that are associated with antibiosis activity. These proteins accumulate in the seed tissues and are a product of an orthologous multigene family that has evolved through extensive duplication events, and has been co-opted for the purpose of plant defense against bruchids (Sparvoli et al. 2001; Lioi et al. 2003). As seed storage proteins, they accumulate in cotyledons and provide a reserve for amino acids required in seed germination, and seedling development. Phytohaemagglutinin (PHA) is the major lectin of beans. PHA functions as a carbohydrate binding protein that defends plants against predation by most organisms, but is less effectively against cowpea weevil-

Collosobruchus maculatus (Murdock et al. 1990). Yet PHA may have a synergistic effect when combined with other antinutritional storage proteins in inhibition activity to predatory insects. PHA is an antinutritional factor for mammals because it binds to the glycoproteins that line the intestinal tract thus inhibiting nutrient absorption.

Homologous to lectins is a family of arcelins and α-amylase inhibitors (α-AIs) (Chrispeels and Raikhel, 1991). Polypeptides produced from members of this gene family share 45-85% amino acid sequence with lectins, and possess similar tertiary structure. The compounds have been alternatively termed isolectins (Pratt et al. 1990). The PHA-arcelin-α-AI complex locus is among the LLPs that have arisen by duplication of a single gene for an ancestral lectin (Nodari et al. 1993; Van Damme et al. 1998; Sparvoli et al. 1998; 2001). LLPs are also found in certain accessions of lima and tepary bean. These have been described as arcelin-like (AL) and alpha amylase inhibitor-like (α-AIL) proteins (Mirkov et al. 1994; Sparvoli et al. 1998; 2001; Lioi et al. 1999; 2003). In general, variants of PHA and α- amylase inhibitor proteins are widespread, being found in all wild and cultivated genotypes of the genus *Phaseolus*. Conversely, arcelins and arcelin-like proteins have only been found in certain accessions of wild common and tepary bean (Osborn et al. 1986; 1988a; Shade et al. 1987; Mirkov et al. 1994; Acosta-Gallegos et al. 1998; Sales et al. 2000; Lioi et al. 2003). The differential expression of lectins and lectin-like seed proteins among legumes demonstrates a divergence in function that is related to the evolution of legume species (Lioi et al. 2006).

Antinutritional factors are found in significant quantity in seeds of tepary beans (Campos et al. 1997) and play an antinutritional role against bruchids. Inhibition of protein digestion due to high accumulation of protease inhibitors in seeds of tepary beans

has been illustrated in mice feeding experiments (Idouraine et al. 1992; Magdi et al. 2003). Similarly, protease inhibitors in bruchids were suggested as potential antinutritional deterrents to larvae of *A. obtectus* and result in delayed growth and development (Campos et al. 2004).

Phytohaemagglutinins (PHA)

Legume PHA is made up of variants of tetrameric protein polypeptides that assemble to form a 120 kDa molecule which is processed into smaller subunits of 28-45kDa. It is built from major polypeptides of PHA-E and PHA-L. PHAs occur in 90% of all accessions of common bean. PHA-E causes agglutination of erythrocytes while PHA-L isoforms cause leucocyte agglutination, have mitogenic properties, and bind glycan complexes. The two isoform subunits are produced by tandemly linked genes that share 82% amino acid sequence identity. They make up a series of five isolectins whose biochemical properties have been characterized (Rudiger and Gabius, 2001). The PHA loci from *Phaseolus* and *Vigna* revealed a distant phylogenetic relationship, with divergence leading to profound differences in protein conformation in each species (Zink et al. 1994). The large variability in conformational structure may reflect evolutionary selection pressures conferred by different seed pests of these species.

Antinutritional activity was demonstrated in rats when PHA was fed in either purified form or as bean seeds (Pusztai et al. 1979; 1983; Bollini et al. 1999). PHA inhibits the absorption of nutrients across the intestinal wall because it binds carbohydrates in the intestinal mucosa. Insecticidal properties of PHA were previously described by Janzen et al. (1976). Gatehouse et al. (1984) demonstrated an antibiosis effect to cowpea weevil (*Collosobruchus maculatus*), although these findings were later

disproved by Murdock et al. (1990) and Huesing et al. (1991) using purified PHA in bruchid feeding trials. Other studies (Idouraine et al. 1992; Magdi et al. 2003) demonstrated that the inhibitory activity to mice in flour of tepary bean seeds is due to a high concentration of both PHA and trypsin inhibitors in raw seeds, although PHA induced carbohydrate binding activity, alone it did not cause antibiosis in cowpea weevils. PHA polypeptides as part of the APA locus may enhance the activity of lectin-like proteins against bean bruchids. For example, a wild common bean accession G02771 (Goossens et al. 2000) has the entire APA locus (Kami et al. 2006) and is highly resistant to bruchids.

Alpha amylase inhibitors

α-AIs are another member of the LLPs in the APA locus that accumulate in the seeds as feeding deterrents. α-AIs of beans prevent α-amylase activity of some bruchid species and other insect species, as well as some mammals. α-AIs inhibit starch digestion causing carbohydrate starvation, retarded instar development and some times larval mortality for affected bruchid species.

α-AIs of common bean were first identified and described as lectin-like proteins (Moreno and Chrispeels, 1989) associated with plant defense activity. Eight variants have been identified in common bean based on their electrophoretic mobility and inhibitory activity against porcine pancreatic α-amylase and from larval α-amylase of Mexican bean weevil (Ishimoto et al. 1995). Wild accessions of common beans accumulate different variants of α-AIs (Suzuki et al. 1993; 1994; Ishimoto et al. 1995). Major variants that adversely affect bruchids are α-AI-1 and αAI-2. The two variants are allelic,

with αAI-1 being closely linked with PHA and found in both wild and cultivated accessions of common bean. α-AI-2 is only found in wild accessions of common bean and is normally linked to specific arcelin variants (Suzuki et al. 1995). The two α-AI variants have different specificities to bruchids (Ishimoto and Chrispeels, 1996; Morton et al. 2000). Bean genotypes with α-AI-2 provide higher levels of resistance to bruchids compared to α-AI-1. Genotypes containing variants of α-AI-1 and α-AI-3 lack arcelin alleles and confer weak antibiosis to only some bruchid species. α-AI-1 has inhibitory activity towards porcine pancreatic amylase and the α–amylases of *Callosobruchus maculatus*, and *C. chinensis*, but it does not inhibit the α–amylases of *Z. subfasciatus*. α-AI-2 demonstrated resistance to proteolytic digestion and strong inhibitory activity against the amylases of *Z. subfasciatus* (Suzuki et al. 1993; Ishimoto and Chrispeels 1996; Finard-Filho et al. 1996; Grossi de Sá et al. 1997, Franco et al. 2002). Recent studies based on transgenic plants with high expression of α-AI-2 indicated that this protein is not the sole mechanism for resistance to *Z. subfasciatus* (Nishizawa et al. 2007). This indicates the importance of transfer of the entire APA locus block in order to induce strong resistance to bean bruchids. Strong inhibitory activity to bruchid species that specialize on adzuki beans and cowpeas (Ishimoto and Kitamura 1989; Suzuki et al. 1993; 1995; Shade et al. 1994; Schroeder et al. 1995; Ishimoto et al. 1996; Pueyo and Delgado, 1997; Yamada et al. 2005) has been demonstrated in feeding trials with purified α-AIs. However, no inhibition to alpha amylases of *A. obtectus* from purified common bean α-AIs has been observed (Fory et al.1996). Other researchers have reported 40% inhibition of alpha amylases of *A. obtectus* and *Z. subfasciatus* under *in vitro* experiments

using α-AI variants from rye seeds (Iulek et al. 2000; Dias et al. 2005), demonstrating that not all variants of α-AIs are ineffective against bean bruchids.

Other species of *Phaseolus* apart from common bean possess related variants of α-amylase inhibitors that have altered structural conformation and properties. Variant α-AIs have been described in tepary, lima, and scarlet runner bean (Ishimoto and Chrispeels, 1996; Blanco-Labra et al. 1996; Pueyo and Delgado-Salinas; 1997; Lioi et al. 1999). Some isoforms of α-AIs from tepary bean were initially characterized by Blanco-Labra et al. (1996) and Yamada et al. (2005) and demonstrated significant differences in their properties compared to those in common bean. Tepary beans possess significant genetic variation for α-AIs that protect legumes from *Z. subfasciatus* and *C. chinensis*. We expect that the presence of α-AI variants with other homologous lectin-like proteins from wild tepary bean may be effective against a wide range of bruchid amylases.

α-AIs from *P. acutifolius* (α-AI^{pa}) demonstrated amino acid sequence similarity to αAI-2 of wild common bean accessions (Yamada et al. 2001; 2005). Though no inhibition of *A. obtectus* amylases by the α-AIs from tepary bean has been reported, isoforms of α-AI^{pa} from wild tepary bean may inhibit or may act as precursor for other lectin-like proteins that have inhibitory effects (Finard-Filho et al. 1996). Tepary bean may have several variants of α-AI^{pa} in one genotype (Yamada et al. 2001). As part of the APA locus, these are also distributed as tandem loci, with evolutionary divergence over time. We think that novel insect α-amylase inhibitory activity from tepary bean accession G40199 can contribute to useful resistance to bruchids if incorporated into cultivated common bean. Alternatively, inhibitory activity may be more complex, and involve the

interaction of α-AIs with other seed storage proteins responsible for resistance to bruchids.

Arcelins and arcelin-like proteins

Arcelins belong to the lectin-like family of seed storage proteins; they contain polypeptides closely related to PHA and α-AIs. Similar to PHA, arcelins may demonstrate a weak carbohydrate-binding activity. Although homologous to lectins, arcelins have a different intrinsic specificity for complex sugars, which leads to a mechanism of toxicity to bruchids (Fabre et al. 1998), as opposed to the monosaccharide-binding of true lectins. The toxic properties of arcelins may be related to recognition and interaction with glycoproteins and other constituents of the digestive tract membranes, as well as direct binding of arcelins to intestinal cells of the insect (Minney et al. 1990; Fabre et al. 1998; Paes et al. 2000). Arcelins are also resistant to proteolytic cleavage by trypsin, chymotryspsin and pepsin enzymes. Thus larvae feeding on bean seeds will have less protein for development where arcelin is the major source of protein.

Arcelins were first found in a limited number of wild common bean accessions from Mexico (Osborn et al. 1988a and 1988b). They were discovered as a protein that was associated with inhibition of development of some species of bruchids. Approximately 10% of wild common bean accessions from Mesoamerica possess arcelins. In addition to common bean, tepary beans are also known to contain variants of arcelin proteins. This protein is absent in cultivated common bean (Chrispeels and Raikhel, 1991) presumably as a result of arcelins not making it through the domestication bottleneck. Like PHA and α-AIs, arcelins also arose as a result of independent duplication events of lectins, which evolved into a number of variants. In bean genotypes

with high arcelin levels, they normally replace a proportion of phaseolin (Osborn et al. 1986 and 1988a; Minney et al. 1990; Hartweck and Osborn, 1997). This implies that seed that accumulate large quantities of arcelins or variants are likely to be more resistant to bruchid predation.

Based on amino acid sequence, seven arcelin variants have been described from various accessions of wild common bean. They can be distinguished by distinct electrophoretic polypeptide profiles that range from 31 to 40 kDa in size. Genetically, the variants are different alleles of the same locus discovered by different researchers: *Arl-1, Arl-2, Arl-3, and Arl-4* (Osborn et al. 1986; 1988b; Hartweck et al. 1991), *Arl-5* (Lioi and Bollini, 1989; Goossens et al. 1994) *Arl-6* (Santino et al. 1991) and *Arl-7* (Acosta-Gallegos et al. 1998). Of the seven variants, six can be grouped into three clusters based on cDNA sequence homology (Sparvoli and Bollini, 1998; Sparvoli et al. 2001). *Arl-1*, *Arl-2* and *Arl-6*, share the same cluster while a second group is composed of *Arl-3* and *Arl-4*, the most ancient variants. *Arl-5* with isoforms 5a and 5b forms a separate branch. Further analysis of the genes in the complex APA locus for LLPs have indicated that more than one allele of arcelin variants may be present at the locus (Lioi et al. 2003) and that the complex locus containing variants for *Arl-3* and *Arl-4* may express other arcelin and lectin-like proteins such as α-AIs and PHA.

Bean seeds containing different variants of arcelins demonstrate different levels of resistance to bruchids manifested by delay in days to adult emergence, reduced adult weight as well as a reduction in number of adults emerged as demonstrated in bruchid feeding trials with different bean accessions (Cardona et al. 1990; Kornegay and Cardona 1991; Kornegay et al. 1993; Acosta-Gallego et al. 1998). These researchers found that

some arcelin variants are more effective than others against bruchids, where bean genotypes containing *Arl-1*, *Arl-2*, *Arl-3* and *Arl-4* demonstrated variable antibiosis effect to *Z. subfasciatus* as shown by delayed larvae emergence, reduced weight and longevity of the insect, as well as decreased fecundity. Different arcelin variants from wild common bean accessions have been backcrossed into cultivated lines to improve resistance to bruchids (Osborn et al. 1988b; Cardona et al. 1990; Kornegay et al. 1993; Hartweck et al. 1997). Of the seven arcelin variants, only lines containing *Arl-1*, *Arl-2*, *Arl-4* and *Arl-5* demonstrated moderate resistances to *A. obtectus* (Kornegay and Cardona 1993; Fory et al. 1996; Paes et al. 2000) while genotypes containing other arcelin variants conferred no resistance to *A. obtectus*.

Arcelin variants are not restricted to wild accessions of *P. vulgaris*, because arcelins and arcelin-like proteins have also been described in accessions of tepary bean (Mirkov et al. 1994; NCBI -AF255724; Sales et al. 2000). Related to arcelins are the arcelin-like (AL) seed storage proteins found in cultivated and wild accessions of tepary and lima bean (Lioi et al. 1999). AL of lima beans (*P. lunatus*) was associated with resistance to *A. obtectus*, but no report on the effect of tepary bean AL to *A. obtectus* has been described. Though reported to be present in cultivated tepary bean, arcelin and arcelin-like proteins contribution to resistance to bruchids has not been clearly elucidated. This reputed bruchid resistance residing in tepary bean (that may be associated with the AL proteins) remains to be transferred into common bean cultivars.

CHARACTERIZATION AND IDENTIFICATION OF SEED STORAGE PROTEINS

Different methods are used in identification and characterization of seed storage proteins of beans. Common methods include: protein chemistry for purification of storage proteins for further structural and functional studies, proteomics protocols, and electrophoretic and immunoassay techniques. One of the most common methods has been to electrophorese total seed storage proteins to reveal the molecular size of different seed proteins. One or two dimensional sodium dedosyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) techniques have been extensively used in seed protein characterization to distinguish bean genotypes from the Andean center of domestication from those of the Mesoamerican center of domestication based on electrophoretic mobility of phaseolins (Gepts et al. 1986; Gepts and Bliss, 1986; Kami et al. 1995). Electrophoretic variants of lectins and LLPs have been identified and characterized by SDS-PAGE. Polymorphic electrophoretic protein profiles have been used as markers in selection of breeding lines. Upon electrophoretic separation, phaseolins have a molecular size of 45-51 kDa while those of PHA are between 33-41 kDa (Brown et al. 1981). Conversely, LLPs including arcelins and α-AIs have electrophoretic motilities with size range from 18-33 kDa (Hartweck et al. 1991; Lioi et al. 2003; Yamada et al. 2005). In addition, other low molecular size seed proteins that may play an insecticidal role, such as trypsin inhibitors and other protease inhibitors as well as phytic acids are abundant in tepary bean seeds (Idouraine et al. 1992; Magdi et al. 2003; Campos et. al. 2004) and have been identified from protein electrophoretic patterns and using immunoassays. Where specific or polyclonal antibodies are available, western-blots can be used as a step to confirm the specificity and identity of seed protein within a given protein band. PHA

can also be identified by demonstration of strong agglutination of blood cells and α-AI can be identified by their inhibition activity of the enzyme alpha amylase in a starch solution.

Protein chemistry has been used in identification, purification and quantification of lectins and LLPs from total seed storage proteins (Vargas-Albores et al.1987; Osborn at al. 1988a; Pratt et al. 1990). Protein purification is necessary where a large quantity of a protein is required for making artificial feeding substrates for animal and insect feeding trials and where quantification of the specific proteins in a given genotype is needed. General protein chemistry procedures for protein extraction, purification and characterization are described in Walker (1996) and Ahmed (2005).

Proteomic analysis involving sequencing short N-terminal peptide sequences obtained from trypsin-digested polypeptides, which are then subjected to tandem mass spectrometry (MS-MS) analysis (Liebler 2002; Bienvenut et al. 2005). This type of analysis allows identification of protein subunits from purified sources, or directly from purified gel-electrophoresis bands. Proteomic characterization using MS-MS is a powerful technique for identification of protein mixtures since several specific peptides from protein variants found in a single electrophoretic band can be identified (McCormack et al. 1997; Bienvenut et al. 2002). This technique also facilitates identification of protein variants with related protein families by DNA sequence match to peptide sequences generated by MS-MS analysis (Yates et al. 1995; Bienvenut et al. 2005). Peptide amino acid sequences can be related to DNA sequence, and can be matched with translated sequences from genomic DNA, mRNA or ESTs. This method

can substitute for the use of antibodies where monoclonal antibodies are not readily available, or they are not specific enough to distinguish among related protein variants.

Lectins and LLPs have also been characterized at the DNA level, and sequence information has been deposited in databases. Sequence data are available for genomic DNA, cDNAs from messenger RNA (Osborn et al. 1988b; Mirkov et al. 1994; Yamada et al. 2001, 2005) as well as expressed sequence tags (EST). Recent genomic analysis of the APA locus for *Phaseolus* beans provides a useful bench mark for understanding the allelic composition and genomic sequence of the entire complex locus (Kami et al. 2006).

The techniques outlined above can be used in evaluation of progenies in a breeding program involving the transfer of lectins and lectin-related proteins from donor genotype to cultivars of common beans. Analysis of total seed protein profiles will provide general information of any polymorphism in seed storage proteins between the parents and progenies. Polymorphic electrophoretic protein polypeptides in the progenies will be used for further analysis to identify the specific proteins residing therein by a combination of proteomics, genomic DNA analysis and expression of mRNA for LLPs. Genomic DNA or cDNA sequence alignment to the database sequences for the lectin-like proteins can be used to provide a comparative homology of sequences with the progenies and the donor parents. These are confirmatory techniques that link the presence of gene and the actual expression of proteins (Bahrali and Chrungoo, 2003; Dias et al. 2005; Yamada et al. 2005). Alignment of multiple homologs or orthologs of gene sequences can be used to describe their genetic identity and phylogenetic relationships. Furthermore, protein-peptide sequences from specific bands of the major polymorphic

protein profiles can be matched and mapped on the database of homologous proteins (Bienvenut et al. 2002) that can be directly linked to expression of lectins and LLP genes.

GENETIC RESOURCES OF THE GENUS *PHASEOLUS*

Central Mexico is considered as one of the two main domestication centers for *Phaseolus*. This region represents the highest genetic diversity for wild and cultivated species of beans (Delgado-Salinas et al. 1999). Over 30 species of the genus *Phaseolus* with their origin in Central America have been described (Debouck 1991, 1994). While wild *P. vulgaris* has a high degree of genetic diversity, only a small portion was carried through the domestication process (Debouck, 2000). Wild genotypes from *P. vulgaris* and related species of the genus remain an important gene pool for genetic diversity (Singh, 2001) from where useful agronomic traits can be directly transferred to cultivars of common bean through breeding. A collection of 40,000 accessions of beans are held at CIAT (Centro Internacional de Agricultura Tropical) with about 25,000 and 1,300 accessions of cultivated and wild species of *P. vulgaris*, respectively (CIAT). Over 12,000 accessions of the CIAT bean germplasm are from distant relatives of common bean that include tepary bean genotypes that can be hybridized to selected compatible genotypes of common bean.

Wild bean genotypes may contain qualitative and quantitative resistance for bruchids but as with other traits, bruchid resistance was lost in the domestication bottleneck (Koinange et al. 1996). Various wild common bean accessions are highly resistant to bean bruchids (Osborn et al. 1986, Romero-Andreas et al. 1986; Gepts et al. 1988; Hartweck et al. 1991; Acosta Gallego et al. 1998; Miklas et al. 2006) and have

been used as parents to transfer resistance into cultivated types. Wild tepary accessions have not been extensively explored as a potential genetic resource for bruchid resistance. In addition to tepary bean (Pratt et al. 1990; Munõz et al. 2006), genetic resources for bruchid resistance include scarlet runner bean (*P. coccineus*) (Sicard et al. 2005) and lima bean (Sparvoli et al. 1998; Lioi et al. 1999; 2006). Of these three species, scarlet runner bean is in the secondary gene pool whereas tepary bean is in the tertiary gene pool and lima bean is in the quaternary gene pool of common bean (Debouck, 1999). Interspecific transfer of resistance from scarlet runner bean can be accomplished without special techniques as long as common bean is used as the female parent. Embryo rescue is required to cross common bean with tepary or lima bean. While tepary bean genes have been successfully introgressed into common bean, researchers have never been able to progress beyond sterile F_1 hybrids with common x lima bean crosses (Mok et al. 1978).

BREEDING FOR BRUCHID RESISTANCE

Bruchid resistance is associated with the LLPs and mainly arcelins, although other factors may also be involved. Backcross breeding has been used to incorporate bruchid resistance by incorporating arcelins into cultivated common bean genotypes (Osborn et al. 1988b; Cardona et al. 1990; Kornegay and Cardona, 1991; Kornegay et al. 1993; Misangu, 1997; Hartweck et al. 1991; 1997). Arcelin alleles are inherited as single dominant genes thereby facilitating transfer (Osborn et al. 1988b). *Arl-1* was the first allele introduced into cultivated species (Osborn et al. 1988b) followed by introgression of *Arl-2*, *Arl-3* and *Arl-4* (Romero-Andreas et. al. 1986; Cardona et al. 1990; Hartweck et al. 1991; Kornegay et al. 1993). A breeding strategy to enhance arcelin accumulation also

employs the use of a phaseolin null allele in combination with arcelin (Hartweck and Osborn, 1997). Arcelin accumulates in greater quantities to replace the missing phaseolin, with associated increase in bruchid resistance (Hartweck et al. 1997). While these efforts have created lines with strong *Z. subfasciatus* resistance, they provide only weak to moderate resistance to *A. obtectus* (Cardona et al. 1990; Kornegay and Cardona 1991; Kornegay et al. 1993; Hartweck et al. 1997; Acosta-Gallego et al. 1998; Paes et al. 2000; Sales et al. 2000).

Some accessions of cultivated common bean with moderate levels of resistance to *A. obtectus* and *Z. subfasciatus* were identified by Misangu (1997) and used in breeding programs to generate partially resistant materials adapted to East African production conditions. RAZ lines developed at CIAT and containing *Arl-1* were also used to introgress *Z. subfasciatus* resistance into Tanzanian common bean cultivars seed damage by *Z. subfasciatus* was reduced from 97% to 6.3% in backcrossed Tanzanian lines (Misangu, 1997). *Arl-2* and *Arl-4* with phaseolin null genotypes have been used at Oregon State University (Myers et al. 2001) to develop arcelin containing Africa-adapted lines with phaseolin null for enhanced arcelin concentration similar to 'Sanilac' mutant lines lacking phaseolin or phytohaemagglutinins with enhanced arcelin content -SMARC lines (Hartweck et al.1997).

While resistance to *A. obtectus* and *Z. subfasciatus* may be predominantly associated with arcelins, other factors may be involved in bruchid antibiosis. Recent work based on genetic transformation indicated that *Arl-1* and *Arl-5* may not be the only factors associated with high levels of resistance to bruchids (Goossens et al. 2000; Zambre et al. 2005). Alternatively, a multiple or synergistic interaction of arcelin with

other factors may be involved, which would have not been transferred in a transformation process. As such, a breeding approach is more likely to transfer intact resistance, but resistance can be lost if done without selection pressure for the trait of interest.

Wild common bean accession G12952 was originally described as highly resistant to *A. obtectus*. The resistance was associated with the presence of *Arl-4*. However, when hybridized to cultivated common bean, progenies with equivalent resistance to the bruchids could not be produced after three filial generations (Kornegay and Cardona 1991), suggesting that additional regulatory factors conditioned and/or enhanced resistance to *A. obtectus*. Other possibilities may have been that a regulatory factor might have been lost during the backcrossing process, substitution of lectin-like proteins in the APA locus or that enzymes in the backcross parent proteolytically modify *Arl-4* resulting in loss of antibiosis activity.

INTERSPECIFIC HYBRIDIZATION IN COMMON BEAN

Hybridization of common bean to tepary bean is difficult to achieve due to strong reproductive and genetic barriers that exist as a result of genetic isolation and speciation. *P. vulgaris* x *P. acutifolius* interspecific hybrids usually produce very few and small crippled plants with very low or no fertility (Rabakaorihanta et al. 1980). In further crossing efforts, these may require another round of embryo rescue due to meiotic abnormalities that lead to abnormal and nonfunctioning gametes (Rabakaorihanta 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; Munõz et al. 2004). While interspecific hybridization between common bean and tepary bean may present a challenge of

incompatibility through embryo abortion and sterility problems (Homma, 1956; Mok et al. 1978; Rabakoarihanta et al. 1979; Rabakaorihanta et al. 1980; Jung et al. 1992), success of transfer of genetic resistance and overcoming these genetic barriers remain an important conventional breeding option to improve bruchid resistance of common bean cultivars.

Interspecific hybridization with tepary bean has been tried as a strategy to transfer different alleles of agronomic importance into common bean using a combination of modified backcross via in vitro embryo rescue (Mejia-Jiménez et al. 1994). So far, only resistance to common bean blight (*Xanthomonas campestris* pv. phaseoli) has been successfully transferred from tepary to common bean cultivars (Singh and Muñoz, 1999) providing evidence for gene introgression from tepary bean into common bean.

The use of compatible common bean genotypes as a bridging parent to introgress agronomically favorable alleles into adapted varieties has been suggested (Mejia-Jiménez et al. 1994). Only a few selected parental genotypes proved successful in interspecific hybridizations between *P. vulgaris* and *P. acutifolius* (Homma, 1956; Smartt, 1970; Mok et al.1978; Manen, 1978; Pratt, 1983; 1985). Other strategies to improve interspecific compatibility include the use of intraspecific common bean hybrids as female parents with tepary bean as an inbred donor parent (Federici and Waines, 1989). Alternatively, following successful recovery of F_1 plants, a modified backcrossing strategy described as the congruity backcrossing method (CBC) improved hybrid fertility and increased recombination and introgression of donor parent alleles (Mejia-Jiménez et al. 1994; Anderson et al. 1996; Robleto and Ascher, 1996). Further modification of the backcrossing strategy was described by Mejia-Jiménez et al. (2002) as the modified

double congruity backcross (DCBC) and significantly improved the introgression of tepary bean genotypes into common bean using transgenic facilitator genotypes.

Beside the improvement of compatibility of crosses between tepary bean and common bean it is still evident that the procedure is laborious and yet subject to very low introgression of genes from tepary bean as donor parent. In a population of fertile hybrids, it is still very difficult to break or eliminate the high linkage drag from the donor tepary parent, making it difficult to introgress traits that are quantitatively inherited (Munõz et al. 2004). It is therefore evident that the difficulties associated with interspecific hybridization have prevented the transfer of complex traits, such as drought resistance into common bean. It is expected that lectins and LLPs from tepary bean donor (G40199) are qualitatively inherited, and should not be linked to infertility problems commonly observed among interspecific hybrids. A simple backcross or congruity backcross procedure should be sufficient to transfer the trait from tepary to common bean.

A wild tepary bean accession G40199 highly resistant to *Z. subfasciatus* and *A. obtectus* (Goossens et al. 2000; Mejia-Jiménez et al. 2002; Cardona et al. 2005) was chosen as a donor parent for interspecific hybridization to introgress possible plant defense seed proteins into cultivated common bean. In general, the cross between tepary and common bean requires the use of a common bean parent lacking dwarf lethal genes and rescue of immature embryos. Attempts to cross G40199 to Ascher congruency backcross lines (Anderson et al. 1996) were not successful (Myers et al. 2001) as pod and ovule development was minimal and aborted at about 20 days. Mechanism or factors associated with resistance to bruchids in this accession have not been identified. If this

accession contains the entire APA locus, then resistance may be transferred into cultivars of common bean followed by screening for bruchid resistance among interspecific progenies. It is possible that the simultaneous or multifunctional inhibition mechanism (Campos and Richardson, 1983) may apply to this wild tepary bean accession if several insecticidal protease inhibitors, lectins and lectin-like seed proteins reside in this genotype. The 33 kDa seed protein described in G40199 may be related to the molecular size of variants of tepary bean lectin-like seed proteins. The true identity of proteins expressed in this profile is still unknown and it is not clear if these protein subunits could be associated with deterrent activity against bruchids. Though earlier works demonstrated the possible transfer of tepary bean lectins into common bean (Pratt et al. 1983; 984), protein variants from different genotypes may confer a different inhibitory effect to bean weevil. To date, no resistance factor has been transferred from tepary bean into common bean besides common bacterial blight resistance (Singh and Munõz, 1999). In some of the resulting lines, common bacterial blight resistance is not stably transmitted from generation to generation. Likewise, it is unknown if seed proteins can be easily transferred into common bean, and if these will show stable inheritance. If the same or a related locus is found in wild tepary bean accession G40199, deploying this locus into common bean cultivars could contribute to increased and multi-species bruchid resistance. Interspecific transfer of the distinct AL or LLPs found in the wild tepary bean accession G40199 into common bean cultivars may provide an important step toward enhanced resistance to *A. obtectus*.

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CHAPTER 3

Interspecific transfer and inheritance of arcelin-phytohaemagglutinin-alpha amylase inhibitor seed proteins from tepary bean (*Phaseolus acutifolius* A. Gray) to common bean (*P. vulgaris* L.)

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Abstract

P. vulgaris cultivars 'ICA Pijao', 'Rojo', and '5-593' were crossed to two *P. acutifolius* accessions - a wild type G40199 and an F_2 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 remains unknown. Interspecific F_1 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 BC_1F_2 plants were screened for protein phenotype and the inheritance of seed storage protein profiles contributed by the tepary bean parents. Most of the F_1 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 that may contribute to the disputed bruchid resistance mechanism in G40199.

INTRODUCTION

Interspecific hybridization is an efficient strategy to introduce genetic diversity not present in the target crop species, but available in related wild or cultivated species. This is certainly true for common bean (*Phaseolus vulgaris*) where several species have contributed (*P. acutifolius,* and *P. coccineus*) or may potentially contribute (*P. costarisensis*, and *P. dumosus*) useful agronomic traits. Traits of particular interest include biotic resistances and abiotic stress tolerances. These traits are not found in common bean either because unique traits in the related species evolved after the common bean had diverged from a common ancestor or if the trait was present in the ancestral species, but did not pass through the domestication bottleneck. One trait of interest is resistance to bruchids or bean weevils - pests that feed on mature bean seed in storage. Bruchid resistance is found in wild common bean and related species, but apparently did not pass through the domestication bottleneck (Osborn et al. 1988).

Phaseolus acutifolius is a potential source of resistance to diseases and insects, drought and heat tolerance (Singh 2001). 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 are required to obtain viable F_1 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; Munõz 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) increases success in producing viable fertile hybrids and facilitating recombination. Another method that has improved the introgression of tepary genotypes into common bean is the modified double congruity backcross (DCBC) developed by Mejia-Jiménez et al. (2002) using transgenic facilitator genotypes. 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 Munõz 1999).

In the tropics and subtropics, *Zabrotes subfasciatus* (Mexican bean weevil) and *Acanthoscelides obtectus* (common bean weevil) are the two most common bruchid species in bean producing areas, and are major pests of beans in storage. *A. obtectus* predominates in most bean producing countries of Africa, and has the greater genetic variation and a more cosmopolitan distribution than *Z. subfasciatus* (Gonzalez-Rodriguez et al. 2000). The two species have different temperature adaptation, with *A. obtectus* preferring cooler conditions than *Z. subfasciatus*.

Most forms of insect resistance in plants are quantitatively inherited, which may be difficult to combine to reach economically useful levels. In contrast, simply inherited bruchid resistance is found in wild *P. vulgaris* accessions of the Mesoamerican origin. In particular, high levels of resistance to *Z. subfasciatus* and moderate resistance to *A.*

obtectus has been characterized (Cardona et al. 1990; Kornegay and Cardona. 1991; Kornegay et al 1993; Suzuki et al. 1995; Hartweck et al. 1997).

Resistance is associated with lectin-related seed storage proteins (Sales et al. 2000) sometimes referred to as lectin-like proteins or LLPs, and in particular, the arcelins (ARL[1]) (Osborn et al. 1986, Osborn et al. 1988; Lioi and Bolini 1989; Minney et al. 1990; Goossens et al. 1994; Santino et al. 1991 and Acosta-Gallegos et al. 1998), and alpha amylase inhibitor (α-AI) (Fory et al. 1996; Grossi de S á et al. 1997). Along with phytohaemagglutinin (PHA), genes for these closely related seed storage proteins make up the complex ARL-PHA-α-AI (APA) locus. Evolutionarily, PHA appears to be ancestral to the lectin-related proteins. Apparently, multiple duplication events followed by evolutionary divergence in form and function led to the origin of ARLs and α-AIs. In terms of function, PHAs bind carbohydrates, α-AIs bind to amylase proteins, and the biological activity of ARLs is unclear. All function as deterrents to seed predation by insects, mammals and birds.

Seven ARL alleles from *P. vulgaris*, have been described (Osborn et al. 1986; Lioi and Bollini, 1989; Santino et al. 1991; 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. These give varying levels of

[1]Names and gene symbols for arcelins are in need of standardization. While some researchers use the acronym 'ARC' and the symbol *Arc* for arcelins, the official gene symbol is *Arl* because *Arc* was first used in common bean genetics to describe a seed coat patterning trait in the presence of *T* (http://www.css.msu.edu/bic/PDF/BeanGenesList.pdf). To confuse matters further, the acronym 'ARL' has been used to describe arcelin-like lectin-related proteins. In this chapter, we use ARL as an acronym for arcelin and to represent the structural gene. Gene symbols are given the prefix *Arl* followed by the allele number. Where appropriate, a superscript denoting the species is appended. Arcelin-like proteins are abbreviated as 'AL'.

resistance to the two bruchid species with several conferring strong resistance to *Z. subfasciatus*, but only weak to moderate resistance to *A. obtectus*. For example, nearly complete resistance to *Z. subfasciatus* is conditioned by the *Arcelin-1* (*Arl-1*) allele, which has been transferred into breeding lines by researchers at CIAT and into cultivars by Sokoine University of Agriculture in Tanzania (Cardona et al. 1990; Misangu 1997). A long term project supported by the Bean/Cowpea CRSP has been to introgress *Arl-2* and *Arl-4* into Tanzanian cultivars to provide moderate levels of resistance to *A. obtectus*. Accessions with combined ARL and α-AI alleles appear most resistant to bruchids. In addition, while there is an association between the presence of some ARLs and α-AIs and bruchid resistance, Goossens et al. (2000) found that purified protein and transgenic lines containing *Arl-5* did not completely account for the strong resistance found in wild *P. vulgaris* accession G02771. 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. An unusual protease inhibitor not related to lectins found in *P. acutifolius* seed may also condition resistance to bruchids (Campos et al. 2004) and account for part of the resistance seen in some accessions.

While individual lectins and lectin-related proteins have been cloned and expressed in other species, the classical introgression of the complete APA locus from *P. acutifolius* into cultivars of *P. vulgaris* remains undocumented. *P. vulgaris* and *P. acutifolius* α-AI seed proteins have been transformed 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 transformed into *P. acutifolius* (Zambre et al. 2005).

In addition to those found in wild *P. vulgaris* accessions, lectin-related seed 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* lectin-related proteins fed in artificial seeds to *A. obtectus* (Pratt et al. 1990). The wild tepary bean accession G40199 was identified by researchers at CIAT to be highly resistant to *A. obtectus* and *Z. subfasciatus* (Mejia-Jiménez- et al. 2002). Resistance was introgressed into common bean from *P. acutifolius* using the double congruity back cross (DCBC) method. Interspecific hybrid lines demonstrating strong resistance to *A. obtectus* were developed but resulting seeds could not germinate (Cardona et al. 2005). 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 lectin-related seed proteins do 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') for intraspecific genetic studies in *P. acutifolius* is maintained in the Vegetable Breeding and Genetics germplasm collection at Oregon State University. 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 following rescue of F_1 embryos generated from crosses between G40199 used as the pollen parent, and ICA Pijao, Rojo or 5-593. A few interspecific crosses were made between ICA Pijao and F_2s from the cross G40199 x 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 minutes followed by a 5 minute 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 'Sunshine® SB-40 professional' growing mix (http://www.sungro.com). Plants were acclimatized at high relative humidity by covering them with Magenta Boxes (GA-7 SIGMA®) in the greenhouse under natural light and supplemented with 16 hrs of light supplied by 1000 watt high pressure sodium and metal halide lamps; a temperature range of 26-27°C was maintained. Gradual acclimatization of plantlets was conducted by steady opening of the Magenta boxes and application of a 3-4 months slow-release (APEX®) N-P-K (14-14-14) fertilizer. The F_1 plants were grown until flowering then backcrossed as females, with pollen from ICA Pijao because all F_1 plants were highly sterile. Only a limited number of seeds were obtained from BC_1F_1 for further backcross to recover fertility among interspecific hybrids. BC_1F_1 seeds were planted and BC_2F_1 seeds were obtained by a second backcross to ICA Pijao as a pollen parent. Meanwhile, congruency backcrossing was initiated by crossing some BC_1F_1 lines to G40199 and seeds from this cross were replanted for seed increase.

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 xg 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 % 2β -mercaptoethanol, 0.05 % Bromophenol blue) and was heated for 5 min. at 95°C on a heating block. The denatured polypeptides were size-separated by electrophoresis on 12 % SDS-PAGE gels (BIORAD) followed by staining with 0.1 % Coomasie 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 lectin-like proteins 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 x G40199 cross. Introgression of the similar lectin-like seed protein profiles was scored among interspecific F_1 hybrids and in the backcross generations obtained from the crosses between ICA Pijao x 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.

Genomic DNA characterization of parents and hybrids

Genomic DNA was extracted from young leaf tissue of individual plants from the F_3 tepary bean population, F_1 interspecific hybrids, and backcross families of ICA-Pijao x 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 (Anonymous, 2006) 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.oligosetc.com) were used for primer designing, optimization and determination of melting temperature for each forward and reverse primer (Table 3.1).

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 minutes initial denaturation of template DNA at 94°C followed by 35 cycles of 94°C for 30s, 62°C for 40s and 72°C for 60s followed by a 5 minute final extension at 72°C. A total of 20 µl PCR reaction volume containing 15 ng of genomic DNA, 50 mM $MgCl_2$ 2 mM of each dNTPs, 10 µM forward and reverse primers and 1 unit of Taq-Polymerase (PROMEGA). PCR amplified DNA products were separated by electrophoresis on 2 % agarose gels and visualized after staining with ethidium bromide.

Table 3.1 Oligonucleotide sequences specific for genomic DNA sequences of four lectin-like genes in tepary beans.

Target gene	Primer sequence	Expected size (bp)	Accession #	Reference
Arcelin-like (ARL2^{pa})	Forward: 5'GCT TCC TCC AAC TTA CTC TCT AG 3' Reverse: 5'ATG TGG TGT GAT CGG GGA ACT CG 3')	800	AF255724	NCBI^{z} -direct submission
Tepary bean *Arcelin* (ARC^{pa})^{y}	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 et al. 1994
α-AI^{pa}	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 et al. 2001
PHA^{pa}	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 et al. 1994

[y] "pa" superscript indicates species origin, in this case pa = *Phaseolus acutifolius*.

[z] National Center for Biotechnology Information

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^{pa}, PHA^{pa} and α-AI^{pa} primers were excised from the agarose gels 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

Interspecific hybridization

A total of 127 F_1 hybrid plants from the ICA Pijao x G40199 cross were recovered from embryo culture. Of these, 33 developed into mature F_1 flowering plants, which were used in backcrosses that generated 43 BC_1F_1 plants. Eighty-four plants were crippled and chlorotic, and died after they were transferred into soil culture or during acclimatization (Table 3.2). Relatively similar numbers of embryos were rescued from the cross combinations Rojo x G40199 and 5-593 x G40199, but their survival when transferred to the greenhouse was much lower (Table 3.2). None of the Rojo x G40199 crosses flowered because most of the plants were chlorotic and crippled. The interspecific hybrids from 5-593 x G40199 produced four, very dwarf plants that flowered and could be used for backcrossing to ICA Pijao as a recurrent parent,but it was difficult to synchronize the flowering of 5-593 with the hybrids.

Most F_1 plants were completely sterile, and produced several parthenocarpic pods with undeveloped ovules. All interspecific F_1 hybrid plants were highly male sterile and therefore used as female parents for backcrossing to ICA Pijao as the only recurrent parent because other cultivars of common bean parents had performed poorly in the initial interspecific cross.

Table 3.2. 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 BTF_2).

	Cross combination			
	ICA Pijao x G40199	Rojo x G40199	5-593 x G40199	ICA Pijao x BTF_2
	No.			
Embryos developing into plantlets	127	143	118	8
Plants surviving in soil	56	12	36	4
F_1s flowering & used in backcrosses	33	0	4	4
BC_1F_1 seed set on F_1 plants	43	0	1	4

Fertility was improved in the second generation of backcrossing to ICA Pijao as shown by the increased number of seeds per pod and reduced number of parthenocarpic pods (Appendix 3.1) and self fertility was nearly normal after the second backcross. Backcrossing the BC_1F_1 hybrids to G40199 as the pollen source to develop a congruity backcross population produced few seeds because of a high degree of sterility, these seeds were planted and plants used for further backcrossing with interspecific hybrid - LLP introgression lines in order to increase the proportion of genes/loci from accession G40199 among interspecific hybrids.

Inheritance of lectin-like proteins in a *P. acutifolius* background

Genetic inheritance of lectin-like seed proteins was studied by evaluating total seed storage protein profiles from mature seeds of G40199 and Brown Tepary that were visualized by SDS-PAGE. Polymorphic protein bands from the total seed protein extracts were scored indicating one major polymorphic band at approximate size of 33 kDa for a polypeptide present in G40199 but not in Brown Tepary (Fig. 3.1). The polymorphic band corresponds to the molecular size of arcelins/lectin-like proteins in *Phaseolus* beans.

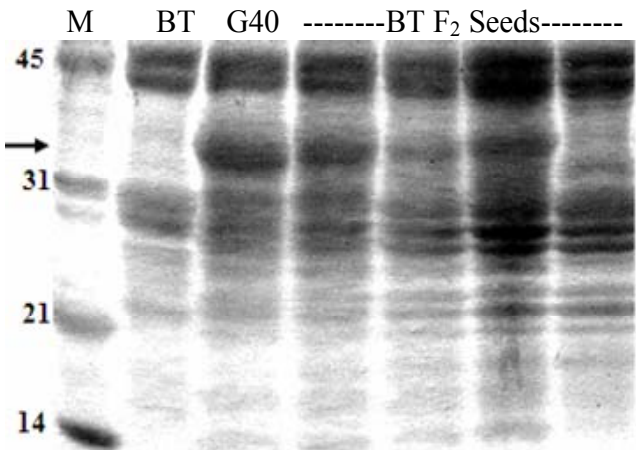

Figure 3.1. Total seed storage protein from mature seeds of Brown Tepary (BT), G40199 (G40), and F_2 intraspecific hybrids (BT F_2 Seeds). Prestained protein standards are shown in column labeled M, and the 33 kDa protein fragment position is marked by the arrow.

Thirty F_3 families were homozygous for the presence of the 33 kDa protein band in the intraspecific cross G40199 x Brown Tepary (BTF_2). Sixty-four families segregated, and 22 families were homozygous for the absence of this seed protein. The segregation ratio for the protein band demonstrated a Mendelian ratio of 1:2:1 for a single dominant allele ($\chi^2 = 3.47$, P = 0.18).

Characterization of lectin-like proteins from interspecific hybrids

Interspecific hybrids were evaluated for the presence of a 33 kDa 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 d embryos taken just prior to embryo rescue for protein determination. A few embryos could be excised and early expression of the 33 kDa protein was observed in some of the interspecific F_1 hybrids (Fig. 3.2).

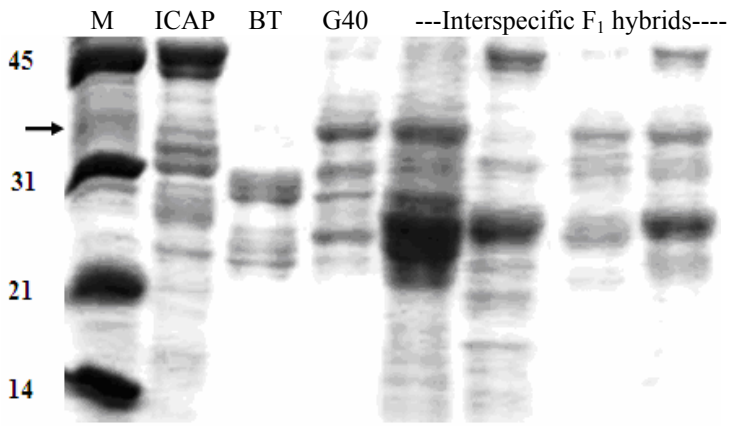

Figure 3.2. Seed storage proteins profiles observed among F_1 interspecific hybrids from ICA Pijao x G40199 cross. Lanes: M, prestained protein standard; ICA Pijao (ICAP); Brown Tepary (BT); G40199 (G40); and interspecific F_1 hybrids. Twenty-two to 28 d old cotyledon tissue was used for interspecific hybrids, mature seed for the other lines. Arrow indicates the position of a ~ 33kDa protein fragment.

The LLP isolated from young cotyledons of F_1 interspecific hybrids was of similar size to that observed in G40199. Following this observation, seeds from interspecific hybrids of the BC_1F_1 and BC_2F_1 were also analyzed for stable introgression and expression of the 33 kDa LLP based on electrophoretic profiles of total seed proteins among backcross progenies (Fig. 3.3). We strongly suspected that 33 kDa protein band was lectin-like protein and possibly an arcelin, warranting characterization at the DNA level.

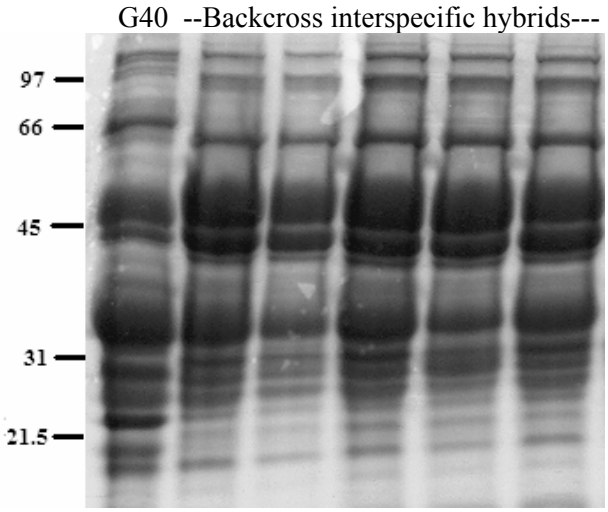

Figure 3.3. Seed storage protein profiles from backcross progenies of interspecific hybrids between G40199 (G40) and ICA Pijao in the BC_1F_1. Backcross interspecific hybrids show stable integration of the ~33kDa protein band. SDS-PAGE standard kDa weights are indicated on the right.

Using the four gene specific primers for arcelins (ARC^{pa} and $ARL\text{-}2^{pa}$), alpha amylase inhibitors ($\alpha\text{-}AIL^{pa}$) and phytohaemagglutinins (PHA^{pa}) for *P. acutifolius*, genomic DNA from accession G40199 demonstrated the presence of $ARL\text{-}2^{pa}$, $\alpha\text{-}AIL^{pa}$ and PHA^{pa} while DNA from Brown Tepary revealed the presence of ARC^{pa} and $\alpha\text{-}AIL^{pa}$ (Fig. 3.4a).

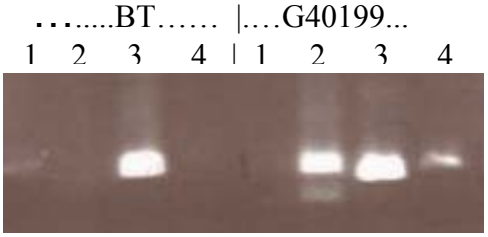

Figure 3.4a: Amplification of ARC^{pa} (1), $ARL\text{-}2^{pa}$ (2), $\alpha\text{-}AI^{pa}$ (3) and PHA^{pa} (4) from genomic DNA of Brown Tepary (BT), and accseeion G40199.

Genomic DNA isolated from intraspecific and interspecific hybrids plants whose seeds had previously revealed the presence of a 33 kDa protein band was subjected to PCR amplification using gene specific primers for ARL-2^{pa}, α-AI^{pa} and PHA^{pa}. A DNA fragment of approximate 790 bp size range was amplified by ARL-2^{pa} primers corresponding to an arcelin-like gene in *Phaseolus acutifolius* (AF255724, NCBI database). A similar size fragment was produced by the same primers in F_1 interspecific hybrids, while no PCR products were generated by these primers in Brown Tepary or ICA Pijao (Fig. 3.4b).

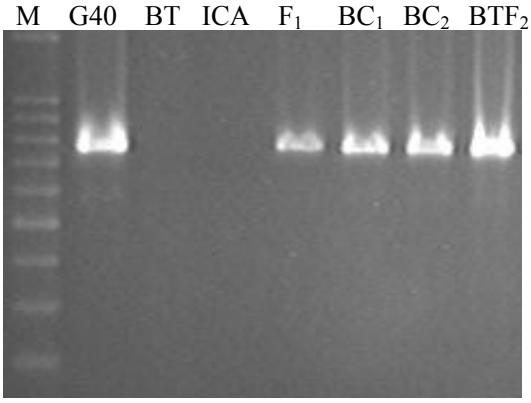

Figure 3.4b. Amplification of ARL-2^{pa} from genomic DNA of G40199 (G40), Brown Tepary (BT), ICA Pijao (ICA) and derived interspecific hybrids (BC_1F_1: BC_1, BC_2F1: BC_2, BT x G40199 F_2). M is a 100bp DNA standard.

In addition, fragments for α-AI^{pa} and PHA^{pa} showed amplification in G40199 and BT but not ICA Pijao (data not shown). All interspecific hybrids containing ARL-2^{pa} also showed amplification of α-AI^{pa} and PHA^{pa}, demonstrating the transfer of the tightly

linked complex locus from G40199 (Fig. 3.5). The DNA fragments for each gene had different molecular size with ARL-2^{pa} at 790 bp, α-AI^{pa} at 750 bp and PHA^{pa} at ~890 bp (Fig. 3.5).

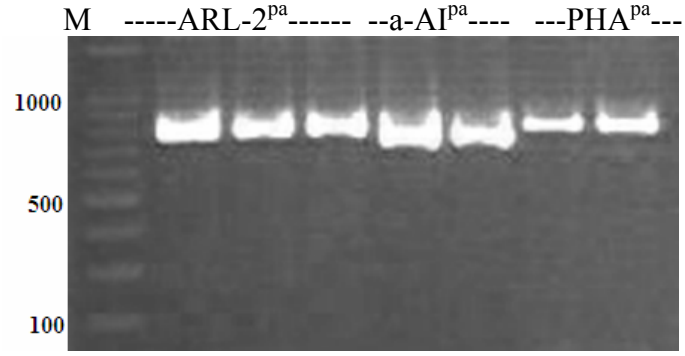

Figure 3.5. PCR amplification of ARL-2^{pa}, α-AI^{pa} and PHA^{pa} from genomic DNA of G40199 (lanes 2, 5, 7), F_1 interspecific hybrid (lane 3), and BC_1F_1 (lanes 4, 6, 8). Lane 1 (M) is a100 bp DNA standard.

Some plants from the Brown Tepary x G40199 F_3 population had PCR products from either ARL-2^{pa} or ARC^{pa} primers but did not segregate for α-AI^{pa}, and PHA^{pa}. Allelic relationships of the two ARL-2^{pa} and ARC^{pa} proteins were analyzed in 60 BC_2F_2 individuals with primers designed for either ARL-2^{pa} or ARC^{pa}. The two alleles were never amplified simultaneously from genomic DNA of homozygous progeny based on screening of 11 plants in each F_3 family (Fig. 3.6) indicating that the two arcelin-like variants may be at the same locus, or in tightly linked loci. In addition, DNA fragments generated by ARC^{pa} primers were never found in interspecific hybrids derived solely from G40199. However, in some interspecific hybrids obtained from three-way cross ICA Pijao x (BT x G40199 F_2), we did observe segregation for the ARC^{pa} fragment

lending support to the idea that ARL-2^{pa} and ARC^{pa} are either alleles at a locus or are tightly linked loci in repulsion, and can only be observed together in the heterozygote.

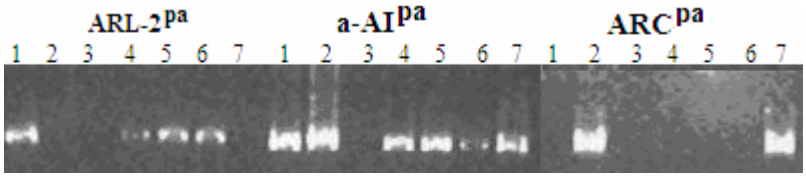

Figure 3.6. Allelic relationship of ARL-2^{pa} and ARC^{pa} and inheritance pattern for α-AI^{pa}. Lane 1, G40199; lane 2, BT; lane 3, ICA Pijao, lanes 4 – 6, BC_2F_2 interspecific hybrids, and lane 7, BT x G40199 F_3 individual that lacked the 33 kDa protein.

Genomic DNA sequencing

Genomic DNA sequences were obtained from PCR amplified products of polymorphic DNA fragments corresponding to ARL-2^{pa}, ARC^{pa} and α-AI^{pa}. These were compared to published sequences for the genes originally used to design primers. DNA sequences for ARL-2^{pa} 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. 3.7).

```
gDNA-ARL2   1   -ASSNLLSRA LFHSASPPTH SQPPHTYFNF DFFKQNDADT NRLILQRDAT ISSGGRLRLS 60
ARL2-pa         MASSNLLSRA LFLLLPTHAI S-ATDTYFNF DFFKQNDADT NRLILQRDAT ISSGGRLRLT
                 ********* **    .. :  * ...***** ********** ********** *********:
gDNA-ARL2   61  GVGSNEDRWV DSMGRAFYSD PIQIRDSTGN LGSFHTNFSF IIRANNGGHS AYGLAFSLVP 120
ARL2-pa         GVGSNEDPWV DSMGRAFYSD PIQIRDSTGN LASFHTNFTF IIRANNAGHS AYGLAFALFP
                ******* ** ********** ********** *.******:* ******.*** ******:*.*
gDNA-ARL2   121 VGSQPKRKRE YLGLFPDAHA VAVAFNTLNN SVDIDVYSYS PSHTGFCDFN KHNGEKTDVQ 180
ARL2-pa         VGSQPKRKRE NLGLFPDAHT VAV-FNTVSN VMKSTSTPTR LAQRGFAIST NHNGETTDVQ
                **********  ********: *** ***:.*  :.    .    :: **.  . :****.****
gDNA-ARL2   181 ITYESPKKNL RVVLHFTKSN VQYEYDFDAP L-LENDVDRS VKRWVGFSAT -GLKEETAET 240
ARL2-pa         ITYESPKKNL KIVLPSTNSN VQ--YDFNAP LYLENEVDRN VS--VGFSAT SGLTEETTET
                ********** ::**  *:** **  ***:** * ***:***. *.  ******  **.***:**
gDNA-ARL2   241 HDILCWSFSS E--------- --------- 270
ARL2-pa         HDILSWSFSS EFPDHTTSEP SNILLNNIL
                ****.***** *
```

Figure 3.7. Deduced amino acid sequence derived from genomic DNA sequence of ARL-2^{pa} from G40199 (gDNA-ARL2) aligned with amino acid sequence from ARL-2^{pa} from an unidentified *P. acutifolius* accession deposited in NCBI database.

Similarly, genomic DNA from the sequenced fragment generated by α-AI[pa] primers in G40199 exhibited 95% nucleotide sequence identity to α-AI[pa] described in tepary bean by Yamada et al. (2001). Genomic DNA sequence from G40199 deduced into amino acid sequence demonstrated 87% identity (Fig. 3.8) to α-AI-1[pa] and 80% sequence identity to α-AI-2[pa] 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.

```
α–AI–pa       1   MASSKFCSVL SLVLFLVLLT HANSACNTSF NFHSFNETNL MLQGQATVSS  50
gDNA-αAI-G40      -SSKSAVSLP SPLPCLVLLT HANSASDT-F NFHSFNETNL ILQGDATVSS
                   :*..  *:  * :  ***** *****.:* * ********** :***:*****
α–AI–pa      51   NGNLQLNTMD SMCSAFYSAP IQIRDSTTGN VASFDTNFTI NMTSYCKANS  100
gDNA-αAI-G40      NGNLQLHTMD SMCSAFYSAP IQIRDSTTGN VASFHTNFTM NITTYRKANS
                  ******:*** ********** ********** ****.****: *:*:* ****
α–AI–pa      101  AVGLDFALVP VQPKSKGRLL GLFKTPDYDR NAGNVTVEFD TFRRRISIDG  150
gDNA-αAI-G40      AVGLDFALVP VQPKSKGRLL GLFKTPDYDR NAGIVTVEFD TLRRRISIDG
                  ********** ********** ********** *** ****** *:********
α–AI–pa      151  NHNDIESVPW DVDDYDGQNA EVRITYNSST KVLAVSLLNL STGKSNNVSA  200
gDNA-αAI-G40      NYNDIESVPW NVDDYDGQKA EVRITYNSST KVLAVSLLNP STGKSNNVSA
                  *:******** :*******:* ********** *********  **********
α–AI–pa      201  RMELEKKLDD WVSVGFIGTS GVHQYSFETR DVFSWSFSSK FSQHTTSERS  250
gDNA-αAI-G40      RMELEKKLDD WVSVGFIGTS GVHEYSFENE RRVLLVFFFV LPTHHIFPT-
                  ********** ********** ***:****..   .   *    :. *
α–AI–pa      251  NILINQIL  258
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Figure 3.8 Amino acid sequence alignment from translated α-AI genomic DNA of G40199 with a complete amino acid sequence for α-AI-1[pa]. * = identical residues, : = similar residues and - indicate gaps.

DISCUSSION

The inheritance of lectin-like genes from tepary bean demonstrated single gene 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 suited 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. It was used for interspecific hybridization in an attempt to transfer the trait directly rather than going through a bridge parent. However, if we had relied solely on Rojo to facilitate interspecific transfer, then the project would have failed. '5-593' was used in crossing attempts because it is a widely used genetic stock, 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 F_1 progeny, it was then used to produce the BC_1 and BC_2 generations. We also developed interspecific hybrids from a three-way crossing and demonstrated introgression of tepary phenotypic

traits and LLPs. We confirmed the stable introgression of LLPs from G40199 among several backcross families.

Polypeptide size of the unique 33 kDa protein revealed by SDS-PAGE in G40199 corresponds to the size of LLPs found in wild common bean and other tepary bean accessions (Pratt et al. 1990, Dillen et al. 1997). Similar-sized seed proteins associated with ARL, α-AI, and PHA have been characterized in some 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.

We did not develop antibodies or screen antibodies for ARL, α-AI or PHA due to the non specific cross-hybridization of these protein epitopes. In-gel peptide sequencing of the polymorphic 33 kDa polypeptide subunit may be an alternative for identification of the candidate protein bands visualized in the SDS-PAGE gel. Alternatively, a genomic DNA analysis approach using oligonucleotide primers for screening of candidate genes takes into account minor variations in nucleotide sequences among the variants of ARL, PHA, and α-AI. Genomic DNA analysis and information from NCBI database for lectin-like genes were used to determine corresponding genes from a family of LLP variants in tepary bean accessions. The unambiguous amplification of polymorphic genomic DNA fragments by gene specific primers directly reflected the presence of a gene in the parent tepary bean and the derived interspecific and intraspecific hybrids. Cosegregation of protein profiles among hybrids and polymorphic amplification of genomic DNA fragments corresponding to AL, ARL, α-AI and PHA variants indicated that the three genes may co-segregate with the 33 kDa protein subunit.

When genomic DNA derived sequences were aligned with nucleotide sequences of genes from database, the accession G40199 was found to contain an arcelin variant with a 94% DNA sequence identity to ARL-2^{pa}, with 72% amino acid sequence identity. The accession that was used to produce the original ARL-2^{pa} 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^{pa} seed storage protein is a functional protein that conditions resistance to bruchids. In designating it "arcelin like" the researchers who deposited the sequence were unsure of whether this gene represented a functional arcelin. We believe that the ARL allele in G40199 is functional based on feeding trials with *A. obtectus* (Chapter 5 this dissertation).

A second gene amplified by primers designed for α-AI^{pa} produced a relatively similar fragment whose nucleotide sequence shared 88% sequence identity to α-AI-1^{pa} described by Yamada et al. (2005) with 79% amino acid sequence identity. α-AI proteins have been shown to inhibit α-amylase activity of *Z. subfasciatus*, but have not been tested against *A. obtectus*. Even so, the successful transfer of this protein into common bean should contribute to additional genetic variability that enhances bruchid resistance if a synergetic mechanism is involved. Genomic DNA from PHA^{pa} 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, accessions 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*. 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 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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CHAPTER 4

Identification of expressed lectin-like protein profiles from tepary bean G40199 and interspecific hybrids and phylogenetic relationship to other bean lectins.

Kusolwa PM and Myers JR

Abstract

Various alleles of arcelin and arcelin-like seed storage proteins confer resistance to *Zabrotes subfasciatus*, but are less effective against *Acanthoscelides obtectus*. Electrophoretic profiles corresponding to lectin-like proteins with 21-33kDa from *Phaseolus acutifolius* accession G40199 were isolated from SDS-PAGE of total seed proteins and subjected to in-gel trypsin digestion, purification and MS-MS peptide sequencing. Peptide mass and amino acid sequences generated from each protein profile were subjected to BLAST search to determine the matching homologous proteins. Meanwhile, cDNA sequences were generated from immature seeds of G40199, Brown Tepary bean, and derived interspecific hybrid backcross lines for analysis of expression of arcelins and arcelin-like genes. ARL-3^{pa} and ARL-4^{pa} cDNA sequences with homology to *P. acutifolius* ARL2^{pa} and ARC^{pa} respectively, were observed in the wild accession G40199 and interspecific hybrids demonstrating the stable transfer and expression of the proteins. Phylogenetic analysis demonstrated the difference of the two arcelin variants in G40199 that clustered with *P. acutifolius* arcelin-like proteins and were separated from those of *P. vulgaris*. Similarly, short peptide sequences from 33 kDa protein profile indicated homology to deduced amino acid sequences of ARL-3^{pa} cDNA and ARL-2^{pa} in G40199 and interspecific hybrid backcross lines. Peptides from protein subunits other than 33 kDa were also identified to be associated with ARL2^{pa}, ARC^{pa} and PHA^{pa} proteins. The stable expression of introgressed tepary bean arcelin-related proteins among interspecific hybrids may play an important role in bruchid resistance when introgressed to cultivars of common bean.

INTRODUCTION

As part of a search for high levels of resistance to bruchids, Cardona et al. (2002) identified the accession G40199 of tepary bean (*Phaseolus acutifolius*), which confers strong resistance to the bruchids; *Acanthoscelides obtectus* and *Zabrotes subfasciatus*. Resistance mechanism(s) residing in this accession were not documented. A major 33 kDa storage protein corresponding to molecular size of lectins was observed in G41099, and was successfully transferred into selected genotypes of common beans by interspecific hybridization via embryo rescue (this dissertation, chapter 3). The protein was hypothesized to be either one or both of the three seed defense proteins of the lectin family - arcelins (ARL), phytohaemagglutinins (PHA) or alpha amylase-inhibitors (α-AI).

Characterization of genomic DNA from accession G40199 revealed the presence of tightly linked genes of the complex arcelin-phytohaemagglutinin-alpha amylase inhibitor (APA) locus expressed as ARL^{pa}, PHA^{pa} and α-AI^{pa} of *P. acutifolius* (this dissertation, chapter 3). Seeds of progeny from interspecific hybrids contained the 33 kDa storage proteins, and the same genes as found in G40199 were found in genomic DNA of interspecific progeny. It was unclear if this complex protein locus alone was associated with the expressions of lectin-like proteins (LLP) and if functional seed proteins for ARL, PHA and α-AI are all confined to this locus. Identification of proteins in the 33 kDa subunit and any other co-segregating protein subunits is necessary in order to associate them with the expression of LLP genes observed in G40199 and its interspecific hybrids. Some or all of the APA proteins in the 33 kDa fragment may be associated with bruchid

resistance as is the case of other APA containing wild common bean genotypes (Goossens et al. 2000). Protein peptide identification and early expression of the seed proteins in the parent G40199 and its derived interspecific hybrid backcross lines is necessary in order to identify any modifications of gene expression between the parent and interspecific hybrids.

Lectin seed proteins of *P. acutifolius* have been associated with resistance to bruchids (Pratt et al. 1990) where high accumulation of PHA was implicated. Mirkov et al. (1994) and NCBI Anonymous (2006) described arcelin and ARL-2 protein variants differing slightly in amino acid sequence but otherwise homologous to *P. vulgaris* arcelins. In addition, tepary beans contain α -AI proteins (Yamada et al. 2001; 2005) with close homology to α -AI-2 from *P. vulgaris*. Their expression in *P. acutifolius* seeds is associated with resistance to *Z. subfasciatus*. However, further evaluations with transgenic leguminous plants expressing individual genes for α -AI-1^{pv}, α -AI-2^{pv}, or arcelins alone in their seeds indicated that these proteins are not alone responsible for resistance to bruchids (Goossens et al. 2000; Zambre et al. 2005; Nishizawa et al. 2007). As such, resistance to bean weevils may be linked to the simultaneous expression of more than one or all of the genes in the APA locus (Kami et al. 2006).

In G40199, it has not been clear if the APA locus genes are transcribed and expressed as intact and functional proteins and whether post-translational alterations may occur. In addition, arcelin-like proteins in *P. acutifolius* have previously been described as pseudogenes, and only observed in genomic DNA (Kami et al. 2006). Expressed mRNA sequences need to be related to peptide sequence from the 33 kDa and other homologous protein subunits in G40199 to determine functionality. Analyzing total

mRNA for LLP expressed in immature seeds may identify other arcelin variants that are co-expressed with the 33 kDa proteins. This work reports the composition, genetic expression and phylogenetic relationships of LLPs in tepary bean G40199, interspecific hybrids and a cultivated brown tepary bean.

MATERIALS AND METHODS

Plant materials

Wild *P. acutifolius* accession G40199, cultivated Brown Tepary bean (BT), and backcross inbred lines of interspecific hybrids from the cross between ICA Pijao x G40199 and an inbred 'Rojo' backcross line containing tepary bean introgressed from bridge interspecific hybrid parent were used to study the expression and identity of the lectin-like proteins. The origin of the accessions and development of interspecific lines was discussed in chapter 3. All seeds used for analysis came from plants previously identified to contain PCR amplified DNA fragments for arcelin, phytohaemagglutinins and alpha amylase inhibitors from genomic DNA. Dry seeds were used for total protein SDS-PAGE analysis and peptide sequencing while immature seeds were used for total RNA extraction.

RNA Assays and cDNA development

Total RNA was isolated from 200 mg of young cotyledons of mid-mature seeds 22 days after pollination. The seeds were finely ground in liquid nitrogen using Qiagen-RNeasy plant mini kit protocols as directed in the kit's manual. One µg of the total RNA was subjected to reverse transcription with reverse transcriptase SuperScript III RT (Invitrogen™). First strand cDNA from total RNA was generated by cDNA synthesis kit and subjected to RT-PCR with oligo-dT priming. First strand synthesized cDNA templates were PCR amplified using the gene specific primers as developed in chapter 3 and listed in table 4.1.

Table 4.1 Gene specific primers used to amplify cDNA sequence from *P. acutifolius* G41099 and derived interspecific hybrid *P. vulgaris* lines.

Target gene [z]	Primer sequence	Expected size (bp)	Accession	Reference
Arcelin-Like (ARL2^{pa})	Forward: 5'GCT TCC TCC AAC TTA CTC TCT AG 3' Reverse: 5'ATG TGG TGT GAT CGG GGA ACT CG 3'	800	AF255724	(NCBI[y]) - direct submission
Tepary bean Arcelin (ARC^{pa})	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 et al. (1994)
α-AI^{pa}	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 et al. (2001)
PHA^{pa}	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 et al. (1994)

[z] "pa" superscript indicates species origin, in this case pa = *Phaseolus acutifolius*.

[y] National Center for Biotechnology Information

Resulting fragments from the selective PCR amplification of the first cDNA strands were isolated from 2% agarose gels and purified by gel purification kit (QIAGEN). The gel purified DNA products were subjected to direct DNA sequencing at the Center for Gene Research and Biotechnology (CGRB) at Oregon State University using an ABI 3730 capillary sequence machine. Sequencing was done using separate reactions for forward and reverse primers.

cDNA sequence analysis

Consensus cDNA sequence was generated by cap3 contig assembly program (Huang and Mandan, 1999) using BIO-EDIT software for each gene to evaluate the nucleotide sequence chromatograms for optimization and sequence alignments of products from forward and reverse primers. Contigs for cDNA sequences from G40199, Brown Tepary, and interspecific hybrid backcrosses to ICA Pijao and Rojo were used for BLASTn search of closest homologous genes in the database. Nucleotide sequences were aligned by Clustal-W algorithm at OSU-CGRB bioinformatics programs to determine genetic identity for nucleotide and amino acid residues. Nucleotide sequences from cDNA were translated into six possible translation frames to obtain consensus amino acids sequence using Seq-tool algorithm. Each translation frame of amino acids was subjected to BLASTp search in the web to determine sequence similarity and identity of amino acids to lectins and lectin-like proteins.

Phylogenetic analysis

Nucleotides and amino acid sequences for several accessions of LLPs from *Phaseolus* species (Table 4.2) were used for multiple alignments using Clustal-W program. Amino acid and cDNA sequences for lectin-like proteins from common bean, wild tepary bean G40199, cultivated Brown Tepary bean and interspecific hybrids were used for phylogenetic analysis. PAUP* 4.0 b10 (Swofford, 1998) was used for bootstrap heuristic search and constructing a phylogenetic tree using bootstrap values for 1000 replicates in parsimony optimization criterion. Phylogenetic trees were visualized using TreeView program (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html)

Table 4.2 Lectins and lectin like gene sequences from NCBI[z] and EMBL[y] databases.

Gene	Species origin	Accession No.	Database source
Arc-1	*P. vulgaris*	AAA33752	NCBI
Arc-2	*P. vulgaris*	AAA33754	NCBI
Arc-3	*P. vulgaris*	AJ534654	EMBL
Arc-4	*P. vulgaris*	AAA67354	NCBI
Arc-5-1	*P. vulgaris*	CAA85418	EMBL
Arc-5c	*P. vulgaris*	AAF23725	NCBI
Arc-6	*P. vulgaris*	CAA04960	EMBL
Arc-7	*P. vulgaris*	AJ439566	EMBL
PHA-^{pv}	*P. vulgaris*	CAD29132	EMBL
LEC-^{pv}	*P. vulgaris*	CAD29133	EMBL
PHA -^{pa}	*P. acutifolius*	AAA82181	NCBI
ARC -^{pa}	*P. acutifolius*	AAA67350	NCBI
ARL2-^{pa}	*P. acutifolius*	AF255724	NCBI
α-AI-1-^{pa}	*P. acutifolius*	BAB72258.1	NCBI
α-AI-2-^{pa}	*P. acutifolius*	BAB72259	NCBI
α-AI-^{pv}	*P. vulgaris*	AAA67355	NCBI
α-AI-2-^{pv}	*P. vulgaris*	CAD28839	NCBI

[z] National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/)
[y] European Molecular Biology Laboratory (http://www.ebi.ac.uk/embl/)

Protein band extraction for sequencing

Total seed protein and peptide extraction

Ground cotyledonary bean powder (0.2g) was dissolved in 200µl of extraction solution (0.5M NaCl, 0.25 ascorbic acid pH 2.4) and homogenized by slow and occasional vortex for 30 minutes, the mixture was left to settle at room temperature for 30 minutes then was centrifuged at 14,000xg for 10 minutes.

Salt soluble proteins (10µl) were dissolved in 20µL of 10% SDS buffer, 0.5M Tris HCl pH 6.8, glycerol, 2-β-mercaptoethanol and 0.05% (w/v) Bromophenol blue. The mixture was then heated at 95^{o}C for five minutes to denature the quaternary protein complex into linear primary structure; then cooled at room temperature before loading into 15% Tris HCl, SDS-PAGE (BIORAD^{TM}) for electrophoretic separation of protein polypeptides at constant 150 V for 60 minutes at room temperature. Gels were stained in a 0.1% Coomasie brilliant blue R-250, 40% methanol and 10% acetic acid solution for three hours followed by 1 hour destaining in a solution of 40% methanol and 9% acetic acid to obtain clear profiles of separate protein bands. Destained gels were washed in deionized water for 1hr to remove the remnant acetic acid and methanol before isolation of the Coomasie blue stained LLP protein bands.

The major protein band at approximate 33 kDa molecular size for lectin-like proteins was carefully excised from the gel using a scalpel. Four co-segregating bands of approximately 31 kDa, 28 kDa, 26 kDa and 21 kDa (Fig. 4.1) were also isolated into separate microfuge tubes for in-gel peptide digestion with trypsin. We hypothesized that the polypeptides in these fragments were subunits of ARC^{pa}, ARL-2^{pa}, α-AI^{pa} and PHA^{pa}.

Each band was cut into millimeter cubes and washed twice with 50µL of deionized water for 15 minutes followed by two destaining washes with a 50%/50% solution of acetonitrile/50mM NH_4HCO_3 for 30 minutes to remove the Coomasie blue stain from the gel plug. The gel plug was dehydrated using acetonitrile until the gel turned opaque. Samples were dried for 30 minutes in a speed vacuum centrifuge. The gel plugs were then rehydrated with 50uL of 25mM Ammonium bicarbonate buffer containing 12.5ng/µL trypsin (Promega-Madison WI-USA) pH 8.0 for 45 minutes while chilled on ice to allow the trypsin to infuse into gel plug. Gel plugs were then submerged in excess solution of 25mM ammonium bicarbonate to ensure proper rehydration and subjected to trypsin digestion at 37^oC for six hours in the dark. Trypsin digested peptides were sequentially extracted three times with 50% acetonitrile and the extracts were combined.

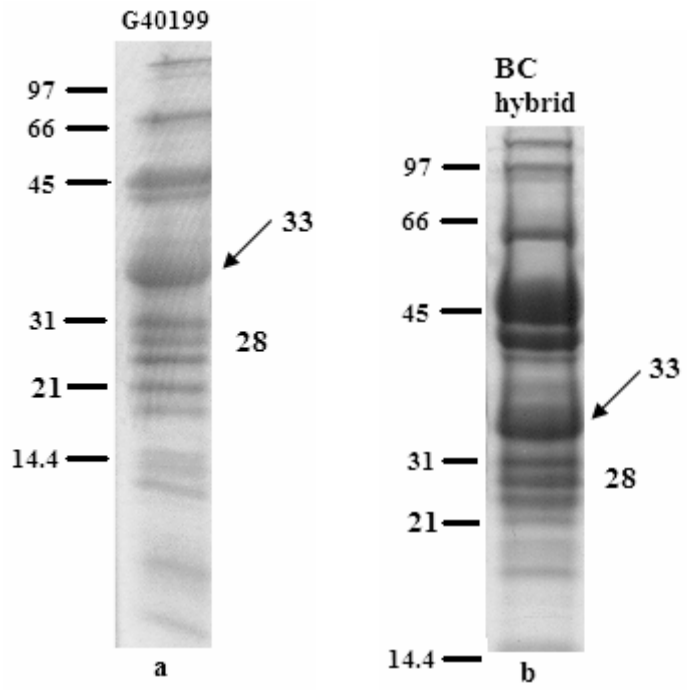


Figure 4.1. Protein profiles from G40199 seed (a) and backcross interspecific hybrid seed (b) as obtained from 15% Tris HCl SDS-PAGE with Coomasie blue R-250 staining. Size standards in kDa indicated to left of gel.

Peptide sequencing

The samples were run by lc/MS-MS using a Waters (Millford, MA) nanoAcquity HPLC connected to Waters Q-ToF-Ultima Global. One-half µl of sample was loaded onto a Waters Symmetry C^{18} trap at 4ul/min, and peptides were eluted from the trap onto the 10cm x 75µm Waters Atlantis analytical column at 350nl/min with solvent A. The HPLC gradient was 2% to 25% of solvent B in 30 min, then to 50% solvent B in 35 min, then 80% B in 40 min and held for 5 minutes. Solvent A was 0.1% formic acid in water, and solvent B was 0.1% formic acid in acetonitrile.

Peptide "parent ions" were monitored as they eluted from the analytical column with half-second survey scans from 400-2000 m/z. Up to 3 parent ions per scan that had sufficient intensity, and had double, triple, or quadruple positive charges were chosen for

MS-MS. The MS-MS scans were 2.4 seconds from 50-2000 m/z. The mass spectrometer was calibrated using the MS-MS spectrum from glu-fibrino-peptide. Masses were corrected over the time the calibration was used (one day or less), using the Waters MassLynx-dxc system.

MS data were processed with Masslynx 4.0 program to produce p^{kl} files, a set of smoothed and centroided parent ion masses with the associated fragment ion masses. Files of parent peptide ions masses and amino acid sequences were searched with Mascot 2.0 (Matrix Science Ltd., London, UK) database software, using mass tolerances of 0.02 for the parent ion and fragment masses. The NCBInr database was used, limiting the searches to plant proteins.

RESULTS

cDNA sequence analysis

Tepary bean G40199

First strand cDNAs produced from total mRNA from immature seeds of G41099, Brown Tepary, and ICA Pijao, and Rojo interspecific backcrosses generated two independent PCR amplified DNA fragments using gene specific primers for ARL-2^{pa} and ARC^{pa} for genes from *P. acutifolius* (Fig. 4.2). ARL-2^{pa} primers produced cDNA amplification of expressed DNA product named here as ARL-3^{pa} (gene symbol *Arl-3^{pa}*) in G40199 genotype and in interspecific hybrids from Rojo and ICA Pijao. Expressed PCR product from ARC^{pa} primers was observed in G40199 as a single DNA fragment with higher molecular weight (~800bp) than the ARC^{pa} cDNA product from Brown Tepary-BT (Fig. 4.2). Amplification of a cDNA product from ARC^{pa} primers in G40199 and interspecific hybrids was not expected because this accession did not produce PCR products from genomic DNA (this work chapter 3). This arcelin product amplified in G40199 by ARC^{pa} primers here in is named ARL-4^{pa} (gene symbol *Arl-4^{pa}*). No cDNA products were detected when gene specific primers for α-AI^{pa} and PHA^{pa} genes were used in PCR.

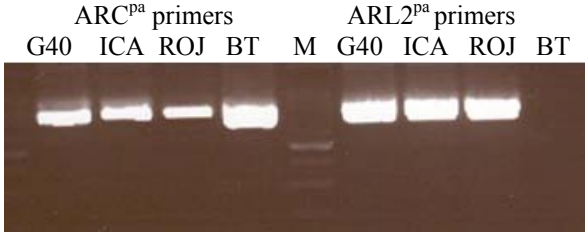


Figure 4.2. cDNA expression of tepary bean ARC^{pa} and ARL-2^{pa} in wild tepary bean G40199 (G40), interspecific hybrids backcross lines (ICA and ROJ) and Brown Tepary (BT) as amplified from total cDNA by PCR using gene specific primers. M = 100bp DNA standards.

Surprisingly, the expression of α-AI^{pa} and PHA^{pa} genes was not observed in 22 d old immature seeds of all the tepary bean genotypes tested, despite the fact that the same primers amplified the gene from genomic DNA. Similarly, the expression of α-AI^{pa} was not evident in total mRNA extracted from immature seeds of derived interspecific hybrids.

The expression of ARL-2^{pa} related cDNA in G40199 was expected because ARL-2^{pa} primers consistently amplified PCR products from genomic DNA in this accession and its derived interspecific hybrids. Nucleotide sequence alignment between ARL-3^{pa} from G40199 cDNA was homologous to ARL-2^{pa} (Fig 4.3) with 95% nucleotide sequence identity consisting of gaps and nucleotide substitutions in relation to ARL-2^{pa} complete DNA sequence (CDs) from (NCBI- AF255724). These results confirm the expression of an arcelin with high similarity to ARL-2^{pa} as a functional gene in G40199 and its derived interspecific lines. ARL-3^{pa} and ARL-4^{pa} sequences generated from the G40199 cDNA do not include the open reading frame for the gene. Nucleotides sequence from ARL-3^{pa} translated into amino acid produced 74% amino acid sequence identity with ARL-2^{pa} (Fig. 4.4).

```
ARL-2pagene        ATGGCTTCCTCCAACTTACTCTCTAGAGCCCTCTTCC-TTCTGCTTC-CCACCCACGCAA  60
ARL-3pa G40199     ---GCTTCCTCCAACTTACTCTCTAGAGCCCTCTTCCATTCTGCTTCTCCACCCACGCAT
                      ********************************** ********* ***********

ARL-2pagene        TCTCAGCCACCG-ACACCTACTTCAATTTCGATTTCTTCAAACAAAACGATGCCGACACA  120
ARL-3pa G40199     TCTCAGCCACCGCACACCTACTTCAATTTCGATTTCTTCAAACAAAACGATGCCGACACA
                   ************ ************************************************

ARL-2pagene        AACAGACTTATCCTCCAACGCGATGCCACCATCTCATCCGGAGGCCGGTTACGACTAACC  180
ARL-3pa G40199     AACAGACTTATCCTCCAACGCGATGCCACCATCTCATCCGGAGGGCGGTTACGACTAAGC
                   ******************************************* ************* *

ARL-2pagene        GGTGTTGGAAGCAACGAAGATCCCTGGGTGGACTCTATGGGCCGCGCCTTCTACTCCGAC  240
ARL-3pa G40199     GGTGTTGGAAGCAACGAAGATCGGTGGGTGGACTCTATGGGCCGCGCCTTCTACTCCGAC
                   **********************  ***********************************

ARL-2pagene        CCCATCCAAATCAGGGACAGCACCGGCAACCTCGCCAGCTTCCACACCAACTTCACATTC  300
ARL-3pa G40199     CCCATCCAAATCAGGGACAGCACCGGCAACCTCGGCAGCTTCCACACCAACTTCTCATTC
                   ********************************** ****************** *****

ARL-2pagene        ATTATCCGCGCTAACAACGCTGGACATTCCGCCTATGGCCTTGCCTTTGCTCTCTTCCCC  360
ARL-3pa G40199     ATTATCCGCGCTAACAACGGTGGACATTCCGCCTATGGTCTTGCCTTTTCTCTCGTCCCC
                   ****************** ***************** ********* ***** *****

ARL-2pagene        GTCGGCTCTCAGCCCAAAAGAAAACGAGAAAATCTAGGTCTTTTCCCCGACGCCCATACT  420
ARL-3pa G40199     GTCGGTTCTCAGCCCAAAAGAAAACGAGAATATCTAGGTCTTTTCCCCGACGCCCATGCT
                   ***** ********************** ************************** **

ARL-2pagene        GTTGCTGTG---TTCAACACCGTCAGCAAC-GTAATGAAATCGACGTCAACTCCAACTCG  480
ARL-3pa G40199     GTTGCTGTGGCGTTCAACACCCTCAATAACAGTGTTGACATCGACGTCTACTCCTACTCG
                   *********   ********* ***  *** **  *** ********* ***** *****

ARL-2pagene        CCTGGCCCAACGAGGTTTTGCGATTTCAACAAACCACAACGGAGAAACGACCGACGTTCA  540
ARL-3pa G40199     CCTTCCCACACGGGGTTTTGCGATTTCAACAAAC-ACAACGGAGAAAAGACCGACGTTCA
                   ***  **  *** ********************** ************ ************

ARL-2pagene        GATCACCTATGAGTCCCCCAAGAAGAACTTGAAGATTGTTCTGCCTTCTACTAATTCGAA  600
ARL-3pa G40199     GATCACCTATGAGTCCCCCAAGAAGAACTTGAGGGTTGTTCTGCATTTCACTAAGTCGAA
                  ******************************** * ********* **  ***** *****

ARL-2pagene        TGTACAGTACGA------TTTCAATGCTCCATTGTACCTGGAGAATGAAGTTGACCGCAA  660
ARL-3pa G40199     TGTACAGTACGAGTACGATTTCGATGCCCCATTGTAGCTGGAGAATGATGTTGACCGCTC
                   ************      **** **** ******** *********** *********

ARL-2pagene        TGTGA------GCGTTGGGTTCTCTGCCACCTCAGGGTTGACGGAAGAGACCACTGAAAC  720
ARL-3pa G40199     GGTGAAGCGTTGGGTTGGGTTCTCTGCCACCTGAGGGTTGAAGGAAGAGACCGCTGAAAC
                    ****      * ****************** ******** ********** *******

ARL-2pagene        GCACGACATCCTCTCTTGGTCTTTTTCTTCCGAGTTCCCCGATCACACCACATCTGAACC  780
ARL-3pa G40199     GCACGACATCCTCTGCTGGTCTTTTTCTTCGGAGTT-----------------------
                   **************  ************** *****

ARL-2pagene        TTCCAACATCCTCCTCAACAATATCCTCTAG  810
ARL-3pa G40199     -------------------------------
```

Figure 4. 3. Sequence alignment for ARL-2^{pa} CDs gene from *P. acutifolius* and ARL-3^{pa} cDNA sequence from accession G40199. * indicates conserved identical base, and -- = gaps.

```
ARL-2paGENE           MASSNLLSRALFLLLPTHAIS-ATDTYFNFDFFKQNDADTNRLILQRDATISSGGRLRLT 60
ARL-3pa-G40199        -ASSNLLSRALFHSASPPTHSQPPHTYFNFDFFKQNDADTNRLILQRDATISSGGRLRLS
                       ***********   .. : * ...********************************:

ARL-2paGENE           GVGSNEDPWVDSMGRAFYSDPIQIRDSTGNLASFHTNFTFIIRANNAGHSAYGLAFALFP 120
ARL-3pa-G40199        GVGSNEDRWVDSMGRAFYSDPIQIRDSTGNLGSFHTNFSFIIRANNGGHSAYGLAFSLVP
                      ******* **********************.******:*******.*********:*.*

ARL-2paGENE           VGSQPKRKRENLGLFPDAHTVAV-FNTVSNVMKSTSTPTRLAQRGFAISTNHNGETTDVQ 180
ARL-3pa-G40199        VGSQPKRKREYLGLFPDAHAVAVAFNTLNNSVDIDVYSYSPSHTGFCDFNKHNGEKTDVQ
                      ********** ********:*** ***:.* :.    .   :: **.  .:****.****

ARL-2paGENE           ITYESPKKNLKIVLPSTNSNVQ--YDFNAPLYLENEVDRNVS--VGFSATSGLTEETTET 240
ARL-3pa-G40199        ITYESPKKNLRVVLHFTKSNVQYEYDFDAPL-LENDVDRSVKRWVGFSAT-GLKEETAET
                      **********::**  *:****  ***:*** ***:***.*.  ****** **.***:**

ARL-2paGENE           HDILSWSFSSEFPDHTTSEPSNILLNNIL 268
ARL-3pa-G40199        HDILCWSFSSE-----------------
                      ****.******
```

Figure 4. 4 Pair wise alignment of deduced amino acid sequences of ARL-3^{pa} from accession G40199 cDNA with a complete amino acid sequence for ARL-2^{pa} gene from *P. acutifolius*. * identical residues, :. similar residues and -- = gaps.

The second arcelin variant ARL-4^{pa} expressed in G40199 cDNA from PCR amplification with ARC^{pa} primers from different accessions of *P. acutifolius* (Mirkov et al. 1994) demonstrated 91% identity to ARC^{pa} nucleotide sequence (Fig. 4.5) and 72% identity at amino acid sequence with ARC^{pa} (Fig. 4.6).

```
ARC^pa          ATGGCTTCCTCCAAGTTACTCTCCCTAGCCCTCTTCCTTCTGCTTCTCACCCACGCAACA 60
ARL-4^pa        ------------------------------TCTTCCTTCTGCTTCTCACCCACGCAACC
                                              **************************

ARC^pa          GCCACCACCTTCGATTTCCCTACCTTCCACAAAGAAGATAAAAACAGACTTATCCT-CCA 120
ARL-4^pa        GACACCTCCTTCGATTTCCCTTTCTTCAAACAAGACGATGCCAACAGACTTATCCT-CCA
                * **** **************  **** *  **** ***   ************** ***

ARC^pa          AGGCAATGCCACCATCTCATCCGGAGGCCGGTTACGACTAACCGGTGTTGGAAGCAACGA 180
ARL-4^pa        AGGATCTGCCACCATCTCATCCGGAGGCCGGTTACGACTAAACGGTGTTGGAAGCAACGA
                ***   ********************************** ******************

ARC^pa          AGATCCCAGGGTGGACTCTATGGGCCGCGCC-TTCTACTCCACCCCCATCCAAATCAGGG 240
ARL-4^pa        AGATCCGTGGGTGGACTCTATGGGCGGCGTC-TTCTACTCCACCCCCATCCAAATGAGGG
                ******  ***************** *** * ********************** ****

ARC^pa          ACAGCACCGGCAACCTCGCCAGCTTCGACAACAAGTTCACATTCAT-TATCCGCGCTAAC 300
ARL-4^pa        ACAGCACCGGCAGGGTCGCCAGCTTCGACACCAAGTTCACATTCAT-TATCCGCGCTAAC
                ************   *************** *************** *************

ARC^pa          AACGCTGGACATTC-CGCCTATGGCCTTGCCTTTGCTCT--CGTCCCCGT-CGGCTCTGA 360
ARL-4^pa        AACGTTTGACATTC-CGCCTATGGCCTTGCCTTTGCTCT--CGTCCCCGT-CGGCTCTGA
                **** * ******* ************************  ********* *********

ARC^pa          GCCCAAAAGAAAACAAGAATATCTAGG-TCTTTTCCCCGACGCCCATACTGTTG-TGTGG 420
ARL-4^pa        GCCCAAAAGAAAAGAAGAATATCTAGG-TCTTTTCCCCGACGCCCATACTGTTGCTGTGG
                ************* ************* ************************* *****

ARC^pa          TGTTCAACACCGTCA-GCAACCG-TATTGAAATCGACGTCAACTCCAACTCGCC-TGG-C 480
ARL-4^pa        TGTTGAACACCGTGGTGCAACCGCTATTGAAATCGACGTCAACTCCATCTCGCTGTGG-C
                **** ********   ******* ********************** *****  *** *

ARC^pa          CCAACGAGGTTTTGCGATTTCAACAAACACAACGGAAAA--GACCGACGTTCAGATCACC 540
ARL-4^pa        CCAACGAGGTTTTGCGATTTCAACCCACGCGACGTAAAGACGACCGACGTTGAGATGACC
                ***********************  ** * *** ***   ********** **** ***

ARC^pa          TATGAGTCCCCCAAGAAGAACTTGAGGGTTGTTCTGCATTTCACTAATTCGAATGTAAAG 600
ARL-4^pa        TATGAGTCCCCCAAGAAGAACTTGAGGATGGTTCTGCTTTTCACTAATTCGAATGTACAG
                ************************** * ******* ****************** **

ARC^pa          TACGATTTCAATGCCCCATTGTACCTGGAGAATGATGTTGACCGCTCGGTGAGCGTTGGG 660
ARL-4^pa        TACGATATCAATGCTCCATTGTATCTGGAGAATGATGTTGACCGCAATGTGAGCGTTGGG
                ****** ******* ******** ********************   ************

ARC^pa          TTCTCTGCCACCTCAGGGTTGAAGGAAGAGACCACTGAAACGCACGACGTCCTCTCTTGG 740
ARL-4^pa        TTCTCTGCCACCTCAGGGTTGACCGAAGTGACCAATGAAACGCACCACGTCATCTCTAGG
                **********************  **** ***** ********** ***** ***** **

ARC^pa          TCTTTTTCTTCCAAGTTCGAACCATTCTATGTTAAGGACCAAAAATCTGAAGGTTCCAAC 800
ARL-4^pa        TCTTTTTCTTGCAAGTTCGAATCATTCTATGTTAAGCAGGAAAAATCTGGGAGG------
                ********** ********** ************** *  *********   *

ARC^pa          ATCCTCCTCAACCAAATCCTCTAGA 815
ARL-4^pa        -------------------------
```

Figure 4. 5 Pair wise sequence alignment of ARL-4^{pa} arcelin cDNA from wild accession G40199 with arcelin (ARC^{pa}) mRNA complete sequences for arcelin of *P. acutifolius*. * = identical base, and -- = gaps.

```
ARC-pa              MASSKLLSLALFLLLLTHATATTFDFPTFHKEDKNRLILQGNATISSGGR 50
ARL-4pa G40199      -----------FLLLLTHATDTSFDFPFFKQDDANRLILQGSATISSGGR 50
                               ********* *:**** *:::* *******.********
ARC-pa              LRLTGVGSNEDPRVDSMGRAFYSTPIQIRDSTGNLASFDNKFTFIIRANN 100
ARL-4pa G40199      LRLTGVGSNEDPWVDSMGGVFYSTPIQMRDSTGRVASFDTKFTFIIRANN
                    ************ ***** .*******:*****.:****.**********
ARC-pa              AGHSAYGLAFALVPVGSEPKRKQEYLGLFPDAHTVVWCS-TPSATVLKST 150
ARL-4pa G4019       V-HSAYGLAFALVPVGSEPKRKEEYLGLFPDAHTVAVVLNTVVQPLLKST 150
                    . ********************:************.    *   .:****
ARC-pa              STPTRLAQRGFA-ISTNTTEKTDVQITYESPKKNLRVVLHFTNSNVKYDF 200
ARL-4pa G40199      STPSRCGPTRFCDFNPRDVKTTDVEMTYESPKKNLRMVLLFTNSNVQYDI 200
                     ***:* .   *. :... .:.***::**********:** ******:**:
ARC-pa              NAPLYLENDVDRSVSVGFSATSGLKEETTETHDVLSWSFSSKFEPFYVKD 250
ARL-4pa G40199      NAPLYLENDVDRNVSVGFSATSGLTEVTNETHHVISRSFSCKFESFYVKQ 250
                     ************.***********.* *.***.*:* ***.***.****:
ARC-pa              QKSEGSNILLNQIL 264
ARL-4pa G40199      EKS-GR-------- 256
                     :** *
```

Figure 4. 6. Pair wise alignment of deduced amino acid sequences of ARL-4^{pa} from wild accession G40199 cDNA with a complete amino acid sequence for arcelin (ARC^{pa}) from *P. acutifolius*. * = identical residues, .: = similar residues and -- = gaps.

Interspecific hybrid backcross lines

Immature seeds from interspecific backcross ICA Pijao x G40199, expressed both ARL-3^{pa} and ARL-4^{pa} mRNA as demonstrated by amplification of cDNA products using gene specific primers for the two genes (Fig. 4.2). A Rojo backcross hybrid with the bridge parent demonstrated cDNA expression as well (Fig. 4.2). The cDNA sequences developed from the Rojo BC for ARL-3^{pa} showed 93% nucleotide homology to ARL-2^{pa} (Fig. 4.7) and 82% sequence identity for amino acid sequence (Fig. 4.8). While ARL-4^{pa} expressed in G40199 was also observed in cDNA from interspecific hybrid backcross lines (Fig. 4.2), it was not sequenced.

```
ARL-3pa Rojo         -----TTCC--CAACTTACTCTCTAGAGCCCTCTTCCTTCTGCTTCTCACCCACGCAATC 60
ARL-2pa GENE         ATGGCTTCCTCCAACTTACTCTCTAGAGCCCTCTTCCTTCTGCTTCCCACCCACGCAATC
                          ****  ********************************** *************

ARL-3pa Rojo         TCAGCCACCGACACCTACTTCAATTTCGATTTCTTCAAACAAAACGATGCCGACACAAAC 120
ARL-2pa GENE         TCAGCCACCGACACCTACTTCAATTTCGATTTCTTCAAACAAAACGATGCCGACACAAAC
                     ************************************************************

ARL-3pa Rojo         AGACTTATCCTCCAACGCGATGCCACCATCTCATCCGGAGGCCGGTTACGACTAACCGGT 180
ARL-2pa GENE         AGACTTATCCTCCAACGCGATGCCACCATCTCATCCGGAGGCCGGTTACGACTAACCGGT
                     ************************************************************

ARL-3pa Rojo         GTTGGAAGCAACGAAGATCCCTGGGTGGACTCTATGGGCCGCGCCTTCTACTCCGACCCC 240
ARL-2pa GENE         GTTGGAAGCAACGAAGATCCCTGGGTGGACTCTATGGGCCGCGCCTTCTACTCCGACCCC
                     ************************************************************

ARL-3pa Rojo         ATCCAAATCAGGGACAGCACCGGCAACCTCGCCAGCTTCCACACCAACTTCACATTCATT 300
ARL-2pa GENE         ATCCAAATCAGGGACAGCACCGGCAACCTCGCCAGCTTCCACACCAACTTCACATTCATT
                     ************************************************************

ARL-3pa Rojo         ATCCGCGCTAACAACGCTGGACATTCCGCCTATGGCCTTGCCTTTTCTCTCGTCCCCGTC 360
ARL-2pa GENE         ATCCGCGCTAACAACGCTGGACATTCCGCCTATGGCCTTGCCTTTGCTCTCTTCCCCGTC
                     ******************************************** ***** ********

ARL-3pa Rojo         GGCTCTCAGCCCAAAAGAAAACGAGAATATCTAGGTCTTTTCCCCGACGCCCATACTGTT 420
ARL-2pa GENE         GGCTCTCAGCCCAAAAGAAAACGAGAAAATCTAGGTCTTTTCCCCGACGCCCATACTGTT
                     ************************** ********************************

ARL-3pa Rojo         GCTGTGGCGTTCAACACCCTCAATAACAGTATTGACATCGACGTCAACTCCAACTCGCCT 480
ARL-2pa GENE         GCTGTG---TTCAACACCGTCAGCAAC-GTAATGAAATCGACGTCAACTCCAACTCGCCT
                     ******   ********* ***  *** *** *** ************************

ARL-3pa Rojo         TCCCACACGGGGTTTTGCGATTTCAACAAAC-ACAACGGAGAAAAGACCGACGTTCAGAT 540
ARL-2pa GENE         GGCCCAACGAGGTTTTGCGATTTCAACAAACCACAACGGAGAAACGACCGACGTTCAGAT
                       **  *** ******************** ************ ***************

ARL-3pa Rojo         CACCTATGAGTCCCCCAAGAAGAACTTGAGGGTTGTTCTGCATTTCACTAAGTCGAATGT 600
ARL-2pa GENE         CACCTATGAGTCCCCCAAGAAGAACTTGAAGATTGTTCTGCCTTCTACTAATTCGAATGT
                     **************************** * ********* **  ***** ********

ARL-3pa Rojo         ACAGTACGAGTACGATTTCAATGCCCCATTGTACCTGGAGAATGATGTTGACCGCTCGGT 660
ARL-2pa GENE         ACAGTACG------ATTTCAATGCTCCATTGTACCTGGAGAATGAAGTTGACCGCAATGT
                     ********      ********** ******************** *********   **

ARL-3pa Rojo         GAAGCGTTGGGTTGGGTTCTCTGCCACCTCAGGGTTGAAGGAAGAGACCACTGAAACGCA 720
ARL-2pa GENE         GA------GCGTTGGGTTCTCTGCCACCTCAGGGTTGACGGAAGAGACCACTGAAACGCA
                     **      * ************************** ********************

ARL-3pa Rojo         CG-------CTCCT-----------------------------------------------
ARL-2pa GENE         CGACATCCTCTCTTGGTCTTTTTCTTCCGAGTTCCCCGATCACACCACATCTGAACCTTC 780
                     **       *** *

ARL-3pa Rojo          ----------------------------
ARL-2pa GENE          CAACATCCTCCTCAACAATATCCTCTAG 810
```

Figure 4.7. Nucleotide sequence alignment for ARL-2^{pa} gene complete DNA sequence from *P. acutifolius* and ARL-3^{pa} cDNA sequence from interspecific hybrid backcross Rojo inbred line. * = identical base, and -- = gaps.

```
ARL-2pa_GENE        MASSNLLSRALFLLLPTHAISATDTYFNFDFFKQNDADTNRLILQRDATISSGGRLRLTG 60
ARL-3pa Rojo        ---PNLLSRALFLLLLTHAISATDTYFNFDFFKQNDADTNRLILQRDATISSGGRLRLTG
                       .********** ******************************************

ARL-2pa_GENE        VGSNEDPWVDSMGRAFYSDPIQIRDSTGNLASFHTNFTFIIRANNAGHSAYGLAFALFPV 120
ARL-3pa Rojo        VGSNEDPWVDSMGRAFYSDPIQIRDSTGNLASFHTNFTFIIRANNAGHSAYGLAFSLVPV
                    ********************************************************:*.**

ARL-2pa_GENE        GSQPKRKRENLGLFPDAHTVAV-FNTVSNVMKSTSTPTRLAQRGFAISTNHNGETTDVQI 180
ARL-3pa Rojo        GSQPKRKREYLGLFPDAHTVAVAFNTLNNSIDIDVNSNSPSHTGFCDFNKHNGEKTDVQI
                    ********* ************ ***:.* :.   ...  :: **.  .:****.*****

ARL-2pa_GENE        TYESPKKNLKIVLPSTNSNVQ--YDFNAPLYLENEVDRNVS--VGFSATSGLTEETTETH 240
ARL-3pa Rojo        TYESPKKNLRVVLHFTKSNVQYEYDFNAPLYLENDVDRSVKRWVGFSATSGLKEETTETH
                    *********::**  *:****  ***********:***.*.  *********.*******

ARL-2pa_GENE        DILSWSFSSEFPDHTTSEPSNILLNNIL 268
ARL-3pa Rojo        AP--------------------------
```

Figure 4. 8 Pair wise alignment of deduced amino acid sequences of ARL-3^{pa} cDNA from 'Rojo' interspecific backcross with a complete amino acid sequence for ARL-2^{pa} from *P. acutifolius*. * = identical residues, .: = similar residues and -- = gaps.

Brown Tepary bean

Of all the LLPs tested, only ARC^{pa} was expressed in cDNA of Brown Tepary while ARL-3^{pa}, α-AI^{pa} and PHA^{pa} were not expressed. ARC^{pa} primers amplified a 720 bp fragment from cDNA of immature seeds of Brown Tepary (BT), which was smaller in size compared to the 800 bp ARL-4^{pa} fragment amplified by the same ARC^{pa} primers in G40199 and derived interspecific hybrids (Fig. 4.2). Expressed cDNA nucleotide sequence from the Brown Tepary fragment had 99% nucleotide sequence identity to ARC^{pa} and a 94% identity to ARL-2^{pa}. As such, Brown Tepary actively expresses an arcelin variant (ARC^{pa}) similar to that found by Mirkov et al. (1994) in *P. acutifolius* (Fig. 4.9). Deduced amino acids sequence from Brown Tepary's cDNA sequence demonstrated 86% amino acid sequence identity to ARC^{pa} (Fig. 4.10). No ARL-3^{pa} protein was expressed in Brown Tepary, which was expected because this gene was not present in the genomic DNA.

```
ARC-pa          ATGGCTTCCTCCAAGTTACTCTCCCTAGCCCTCTTCCTTCTGCTTCTCAC  50
ARCpa-BT        ----------------------CCTAGCCCTCTTCCTTCTGCTTCTCAC
                                      **************************

ARC-pa          CCACGCAACAGCCACCACCTTCGATTTCCCTACCTTCCACAAAGAAGATA  100
ARCpa-BT        CCACGCAACAGCCACCACCTTCGATTTCCCTACCTTCCACAAAGAAGATA
                **************************************************

ARC-pa          AAAACAGACTTATCCTCCAAGGCAATGCCACCATCTCATCCGGAGGCCGG  150
ARCpa-BT        AAAACAGACTTATCCTCCAAGGCAATGCCACCATCTCATCCGGAGGCCGG
                **************************************************

ARC-pa          TTACGACTAACCGGTGTTGGAAGCAACGAAGATCCCAGGGTGGACTCTAT  200
ARCpa-BT        TTACGACTAACCGGTGTTGGAAGCAACGAAGATCCCAGGGTGGACTCTAT
                **************************************************

ARC-pa          GGGCCGCGCCTTCTACTCCACCCCCATCCAAATCAGGGACAGCACCGGCA  250
ARCpa-BT        GGGCCGCGCCTTCTACTCCACCCCCATCCAAATCAGGGACAGCACCGGCA
                **************************************************

ARC-pa          ACCTCGCCAGCTTCGACAACAAGTTCACATTCATTATCCGCGCTAACAAC  300
ARCpa-BT        ACCTCGCCAGCTTCGACACCAAGTTCACATTCATTATCCGCGCTAACAAC
                ****************** ******************************

ARC-pa          GCTGGACATTCCGCCTATGGCCTTGCCTTTGCTCTCGTCCCCGTCGGCTC  350
ARCpa-BT        GCTGGACATTCCGCCTATGGCCTTGCCTTTGCTCTCGTCCCCGTCGGCTC
                **************************************************

ARC-pa          TGAGCCCAAAAGAAAACAAGAATATCTAGGTCTTTTCCCCGACGCCCATA 400
ARCpa-BT        TGAGCCCAAAAGAAAACAAGAATATCTAGGTCTTTTCCCCGACGCCCATA
                **************************************************

ARC-pa          CTGTTG-TGTGGTGTTCAACACCGTCAGCAACCGTATTGAAATCGACGTC  450
ARCpa-BT        CTGTTGCTGTGGTGTTCAACACCGTCAGCAACCGTATTGAAATCGACGTC
                ****** ***************************************

ARC-pa          AACTCCAACTCGCCTGGCCCAACGAGGTTTTGCGATTTCAACAAACACAA  500
ARCpa-BT        AACTCCAACTCGCCTGGCCCAACGAGGTTTTGCGATTTCAACAAACACAA
                **************************************************

ARC-pa          CGGA—AAAGACCGACGTTCAGATCACCTATGAGTCCCCCAAGAAGAACT  550
ARCpa-BT        CGGAGAAAAGACCGACGTTCAGATCACCTATGAGTCCCCCAAGAAGAACT
                ****  ***************************************

ARC-pa          TGAGGGTTGTTCTGCATTTCACTAATTCGAATGTAAAGTACGATTTCAAT  600
ARCpa-BT        TGAGGGTTGTTCTGCATTTCACTAATTCGAATGTAAAGTACGATTTCAAT
                **************************************************

ARC-pa          GCCCCATTGTACCTGGAGAATGATGTTGACCGCTCGGTGAGCGTTGGGTT  650
ARCpa-BT        GCCCCATTGTACCTGGAGAATGATGTTGACCGCTCGGTGAGCGTTGGGTT
                **************************************************

ARC-pa          CTCTGCCACCTCAGGGTTGAAGGAAGAGACCACTGAAACGCACGACGTCC  700
ARCpa-BT        CTCTGCCACCTCAGGGTTGAAGGAAGAGACCACTGAAACGCACGACGTCC
                **************************************************

ARC-pa          TCTCTTGGTCTTTTTCTTCCAAGTTCGAACCATTCTATGTTAAGGACCAA  750
ARCpa-BT        TCTCTTGGTCTTTTTCTTCCAAGTTCGAACCATTCTATGTTAAG------
                ****************************************

ARC-pa          AAATCTGAAGGTTCCAACATCCTCCTCAACCAAATCCTCTAGA  793
ARCpa-BT        -------------------------------------------
```

Figure 4. 9. Pair wise sequence alignment of Arcelin cDNA from cultivated Brown Tepary bean with arcelin (ARC^{pa}) complete DNA sequence from *P. acutifolius*. * = identical base, and -- = gaps.

```
ARC-pa          MASSKLLSLALFLLLLTHATATTFDFPTFHKEDKNRLILQGNATISSGGRLRLTGVGSNE 60
ARCpa-BT        --------LALFLLLLTHATATTFDFPTFHKEDKNRLILQGNATISSGGRLRLTGVGSNE
                        ************************************************

ARC-pa          DPRVDSMGRAFYSTPIQIRDSTGNLASFDNKFTFIIRANNAGHSAYGLAFALVPVGSEPK 120
ARCpa-BT        DPRVDSMGRAFYSTPIQIRDSTGNLASFDTKFTFIIRANNAGHSAYGLAFALVPVGSEPK
                ***************************.****************************

ARC-pa          RKQEYLGLFPDAHTVVWCS-TPSATVLKSTSTPTRLAQRGFAISTNTTEKTDVQITYESP 180
ARCpa-BT        RKQEYLGLFPDAHTVAVVFNTVSNRIEIDVNSNSPGPTRFCDFNKHNGEKTDVQITYESP
                ***************.    * *  :  ...: :  . *   :.:. ************

ARC-pa          KKNLRVVLHFTNSNVKYDFNAPLYLENDVDRSVSVGFSATSGLKEETTETHDVLSWSFSS 240
ARCpa-BT        KKNLRVVLHFTNSNVKYDFNAPLYLENDVDRSVSVGFSATSGLKEETTETHDVLSWSFSS
                ************************************************************

ARC-pa          KFEPFYVKDQKSEGSNILLNQIL 263
ARCpa-BT        KFEPFYVK---------------
                ********
```

Figure 4. 10. Pair wise alignment of deduced amino acid sequences from cultivated Brown Tepary bean arcelin ARC^{pa}-BT cDNA with a complete amino acid sequence for arcelin ARC^{pa} from *P. acutifolius*. * = identical residues, .: = similar residues and -- are gaps.

Phylogenetic analysis

The phylogenetic relationship for the ARL-3^{pa}, ARL-4^{pa} and α-AI^{pa} discovered in G40199 and Brown Tepary was compared to nucleotide and amino acid sequences of related LLPs from *P. vulgaris* and *P acutifolius*. ARL-3^{pa} expressed in G40199 and derived interspecific hybrids clustered with ARL-2^{pa} gene previously deposited in NCBI database (Fig. 4.11). The ARC^{pa} reported by Mirkov et al. (1994) was nearly identical to the arcelin found in Brown Tepary. Unlike the Brown Tepary arcelin (ARC^{pa}-BT), the other ARL-4^{pa} allele expressed in G40199 and hybrids fell onto a separate branch from that of ARC-^{pa} of Mirkov et al. (1994). The ARL-4^{pa} has 74% and 62% amino acid sequence homology to ARC^{pa} and ARL2^{pa} respectively (Table 4.3). *P. acutifolius* ARL proteins clustered separately from other lectin-like proteins of *P. vulgaris* showing their divergence from lectin-like proteins of common bean. Arcelin variants of cultivated

tepary beans are more closely related to each other than to those in wild *P. acutifolius* accession G40199 and its derived interspecific hybrids (Fig. 4.12). Arcelins of *P. vulgaris* grouped into three separate clusters: arcelins 1, 2 and 6 together, 3 and 4 in one cluster and 5 and 7 in another cluster. The latter groups seem most closely related to arcelins in *P. acutifolius* (Fig. 4.11).

In contrast to the separate branching for arcelins from different species, the alpha amylase inhibitor from genomic DNA of the *P. acutifolius* genotypes and interspecific hybrids studied here clustered together with the α-AI-^{pa} of tepary bean (Mirkov et al. 1994), the α-AI-2^{pa} (Yamada et al. 2001 & 2005), and the α-AIs of *P. vulgaris* (Fig. 4.11). Sequence similarity of genomic α-AI of Brown Tepary is 79% and 66% similarity to α-AI-1^{pa} and α-AI-2^{pa}, respectively (Table 4.3).

Table 4. 3 Amino acid identity matrix of LLPs from tepary bean and common beans.

Prot Seq	ARC-^{pa}	ARC^{pa}-BT	ARL4^{pa}-G40199	ARL2-^{pa}	ARL3^{pa} Rojo	ARL3^{pa} G40199	αAI-^{pa}	αAI2-^{pa}	α-AI-BT	PHA-^{pa}	Arc5-Pvu	Arc4-Pvu	Arc2-Pvu	Arc1-Pvu
ARC-^{pa}	ID													
ARC-BT	0.83	ID												
ARL4^{pa}-G40199	0.74	0.72	ID											
ARL-2^{pa}	0.72	0.68	0.62	ID										
ARL3^{pa}-Rojo	0.67	0.76	0.70	0.73	ID									
ARL3^{pa}-G40199	0.67	0.69	0.67	0.71	0.85	ID								
α-AI-^{pa}	0.43	0.41	0.26	0.43	0.38	0.37	ID							
α-AI2-^{pa}	0.41	0.39	0.24	0.42	0.38	0.35	0.78	ID						
α-AI-BT	0.35	0.37	0.26	0.39	0.39	0.36	0.79	0.66	ID					
PHA-^{pa}	0.46	0.43	0.29	0.45	0.41	0.38	0.52	0.50	0.46	ID				
Arc5-Pvu	0.59	0.58	0.35	0.55	0.54	0.52	0.51	0.47	0.44	0.48	ID			
Arc4-Pvu	0.34	0.32	0.19	0.35	0.33	0.31	0.33	0.33	0.36	0.35	0.43	ID		
Arc2-Pvu	0.55	0.54	0.33	0.53	0.51	0.48	0.55	0.52	0.50	0.53	0.61	0.44	ID	
Arc1-Pvu	0.35	0.33	0.19	0.35	0.33	0.31	0.34	0.34	0.32	0.34	0.39	0.61	0.52	ID

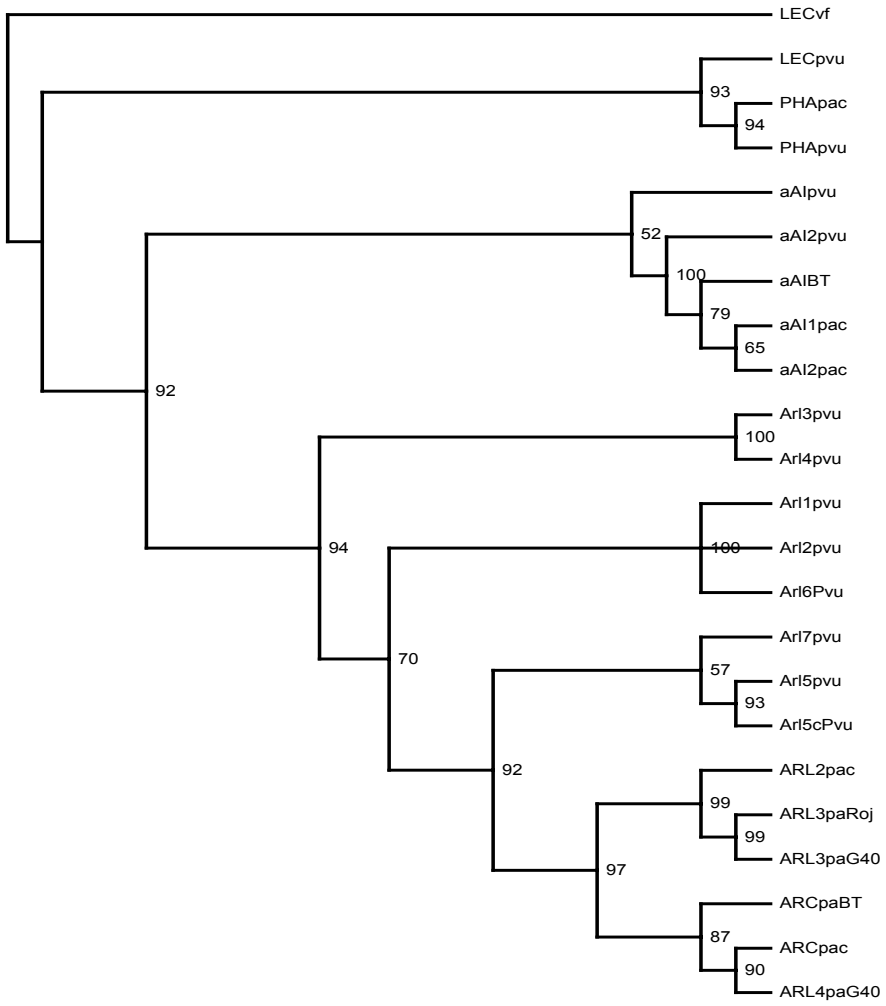


Figure 4. 11. Phylogenetic tree developed from PAUP* program with bootstrap values calculated based on the nucleotide sequences of lectin-like genes from common bean and tepary bean and cDNA sequences generated from G40199, Brown Tepary and the interspecific hybrid backcross line to 'Rojo' containing PHA^{pa}, ARL^{pa} and α-AI^{pa}. *Vicia faba* lectin (LECvf) was used as an out-group. Tepary bean and common bean genes were taken from NCBInr database. Bootstrap values for each node from 1000 replicates are indicated. pac, pvu and vf indicates species *Phaseolus acutifolius*, *P. vulgaris* and *Vicia faba* respectively.

Sequence alignment of the lectin-like proteins (Fig. 4.12) reveals levels of amino acid similarity and their evolutionary relationships to the ancestral lectins (phytohaemagglutinins). Such an alignment can show incidence of duplications, amino acid substitutions and deletions as revealed by gaps. These alterations may be related to altered functional properties depending on where the change occurs in the molecule. Of importance here are the presence of unique gaps and amino acids substitutions between the arcelins in *P. acutifolius* and *P. vulgaris* that defines their essential molecular differences (Fig. 4.12). Amino acid sequences for arcelin variants in cultivated and wild accessions of tepary beans demonstrate their evolutionary difference by the high rate of sequence substitutions and the presence of a unique gap2 providing a distinctive characteristic of arcelins from tepary beans, the other additional gaps in arcelins of *P. vulgaris* are identitical to tepary arcelins showing divergence from other lectin like proteins (Fig. 4.12).

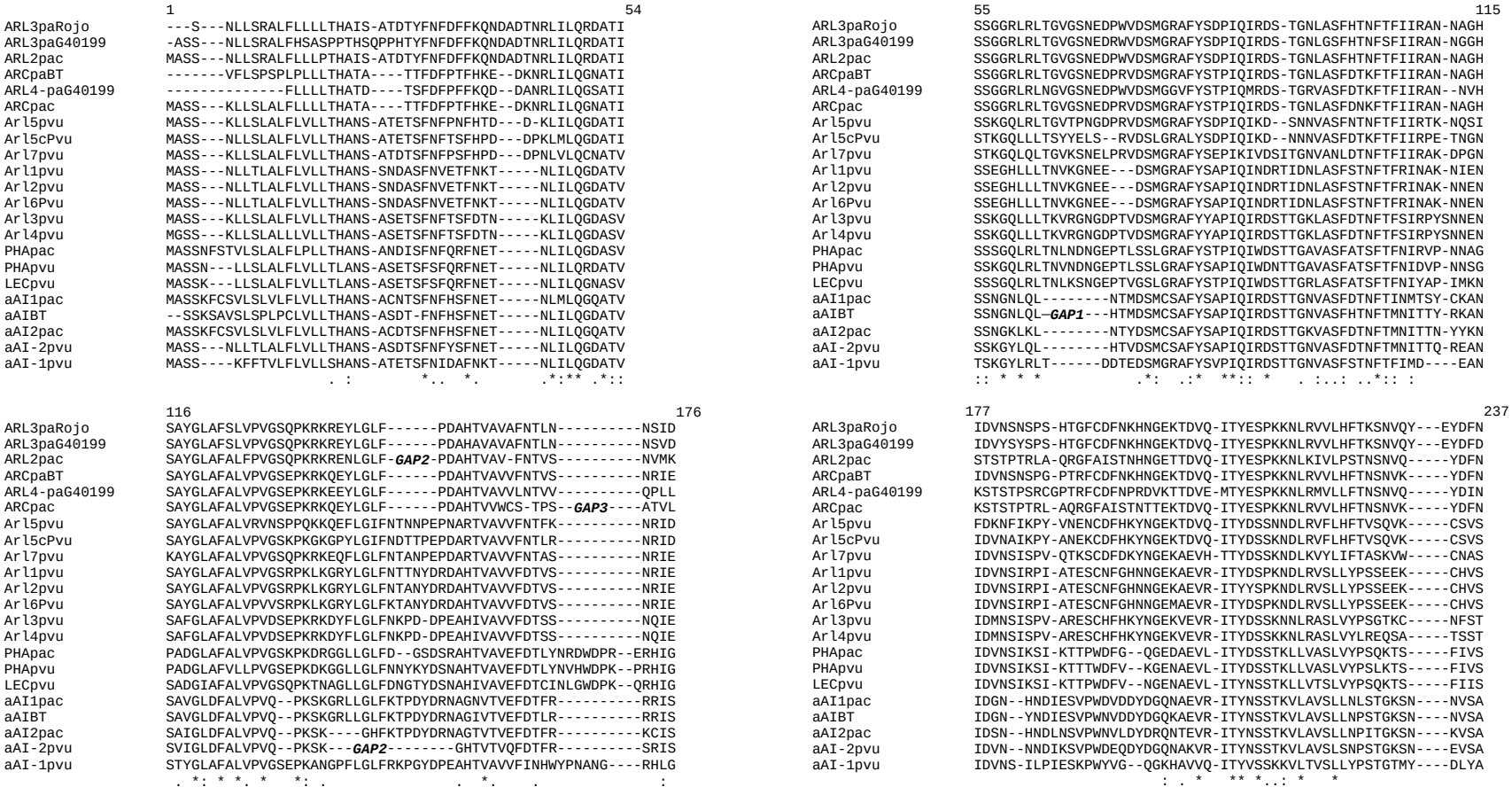


Figure 4. 12 Multiple sequence alignment of arcelins and lectins from *P. vulgaris* and *P. acutifolius*. - - indicates gaps, * = identical amino acids, .: = similar amino acid sequences.

Figure 4.12 (Continued)

```
                 238                                                 298
ARL3paRojo       APLYLENDVDRSVKRWVGFSATSGLKEETTETHAP------------------------
ARL3paG40199     APL-LENDVDRSVKRWVGFSAT-GLKEETAETHDILCWSFSSE----------------
ARL2pac          APLYLENEVDRNVS--VGFSATSGLTEETTETHDILSWSFSSEFPDH-----------TT
ARCpaBT          APLYLENDVDRSVS--VGFSATSGLKEETTETHDVLSWSFSFQVRTI-------------
ARL4-paG40199    APLYLENDVDRNVS--VGFSATSGLTEVTNETHHVISRSFSCKFESFY------------
ARCpac           APLYLENDVDRSVS--VGFSATSGLKEETTETHDVLSWSFSSKFEPF------------Y
Arl5pvu          ATVHLEKEVDEWVS--VGFSPTSGLTEDTTETHDVLSWSFSSKFRNK-------------
Arl5cPvu         ATVQLEKEVNEWVS--VGFSATSGLTENTTETHDVLSWSFSSKFRNK-------------
Arl7pvu          ATVHLEKEVNSWVS--VGFSATSGSKEETTETHDVLSWSFSSKFRNK-------------
Arl1pvu          ATVPLEKEVEDWVS--VGFSATSGSKKETTETHNVLSWSFSSNFINFKGKK---------
Arl2pvu          ATVPLEKEVEDWVS--VGFSATSGSKKETTETHNVLSWSFSSNFINFEGKK---------
Arl6Pvu          ARVPLEKEVEDWVS--VGFSATSGSKKETTETHNVLSWSFSSNFINFKGKK---------
Arl3pvu          SSVHMEKVLNDWVS--VGFSATSGLYDPTSETHDVLSWSFSSKFSQHTTS----------
Arl4pvu          SSVHMEKVLNDWVS--VGFSATSGLYDPTSETHDVLSWSFSSKFSQHTTS----------
PHApac           DTVDLKSVLPEWVR--VGFSATSGITKGNVETNDLLSWSFASKLSDGTTSEG--------
PHApvu           DTVDLKSVLPEWVI--VGFTATTGITKGNVETNDVLSWSFASKLSDGTTSEA--------
LECpvu           DRVELESVLPEWVS--VGFSATSGINEGNTETNDVLSWSFASKLSDGTTSEG--------
aAI1pac          R-MELEKKLDDWVS--VGFIGTSGVHQYSFETRDVFSWSFSSKFS--QHTT---------
aAIBT            R-MELEKKLDDWVS--VGFIGTSGVHEYSFENERRVLLVFFFVLP---------------
aAI2pac          R-MELEKILDDWVS--VGFSATSGAYQWGFETNEVLSWSFSSKFS--QHTT---------
aAI-2pvu         R-MEVEKELDDWVR--VGFSAISGVHEYSFETRDVLSWSFSSKFS--QHTT---------
aAI-1pvu         KKVELEEEVYDWVS--VGFSATSGANQWSYETHDVISWSFSSKFSD-DDDT---------
                   : ::. :   *   ***    *  .   *..
```

```
                 299                 319
ARL3paRojo       ---------------------
ARL3paG40199     ---------------------
ARL2pac          SEPSNILLNNIL----------
ARCpaBT          LCGPKIR---------------
ARL4-paG40199    VKQEKSGR--------------
ARCpac           VKDQKSEGSNILLNQIL-----
Arl5pvu          --LSNILLNNIL----------
Arl5cPvu         --LSNILLNNIL----------
Arl7pvu          --LSNILLNQIL----------
Arl1pvu          SERSNILLNKIL----------
Arl2pvu          SERSNILLNKIL----------
Arl6Pvu          SERSNILLNKIL----------
Arl3pvu          -ERSNILLNKIL----------
Arl4pvu          -ERSNFLLNMFL----------
PHApac           LNLANFVLNQIL----------
PHApvu           LNLANFALNQIL----------
LECpvu           LNLANSLLNQIL----------
aAI1pac          SERSNILINQIL----------
aAIBT            --THHI-FPT------------
aAI2pac          SERSNIVLDKIRSCRLPKPASL
aAI-2pvu         SERSNILLNNIL----------
aAI-1pvu         SERSNILLNNIL----------
```

Peptide sequence analysis

Profiles from total seed storage proteins

The protein profiles from interspecific backcross hybrids were homozygous for the major *P. acutifolius* lectin-like proteins and were consistently stable. While most of the protein subunits from G40199 were observed in the interspecific backcross hybrids, a few were missing or modified (Fig. 4.13). Interspecific backcross hybrids lacked the 26 kDa fragment or had very weak expression compared to G40199 (Fig. 4.13) and a 31 kDa subunit was modified into two subunits of approximately 31.5 and 29 kDa. While the 28 subunit appeared stable among interspecific hybrids as well as in G40199, 21 kDa subunit may have been missing or present but not at the same level as that in G40199 (Fig. 4.13).

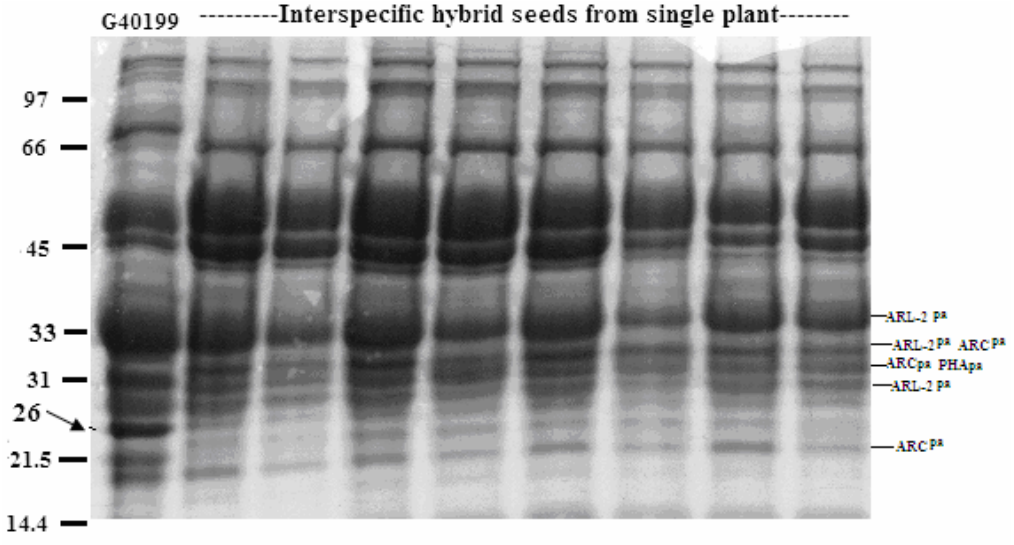


Figure 4.13. Total storage protein profiles from G40199 (lane 1) and interspecific hybrid seeds (each from a BC_2F_3 single plant) separated on 15% Tris HCl SDS-PAGE stained with Coomasie blue R-250. Positions of molecular weight protein standards are indicated at the left. 33 kDa protein subunit and associated lower subunits are shown among interspecific hybrids and the wild tepary bean G40199.

Isolation of peptides and peptide sequencing

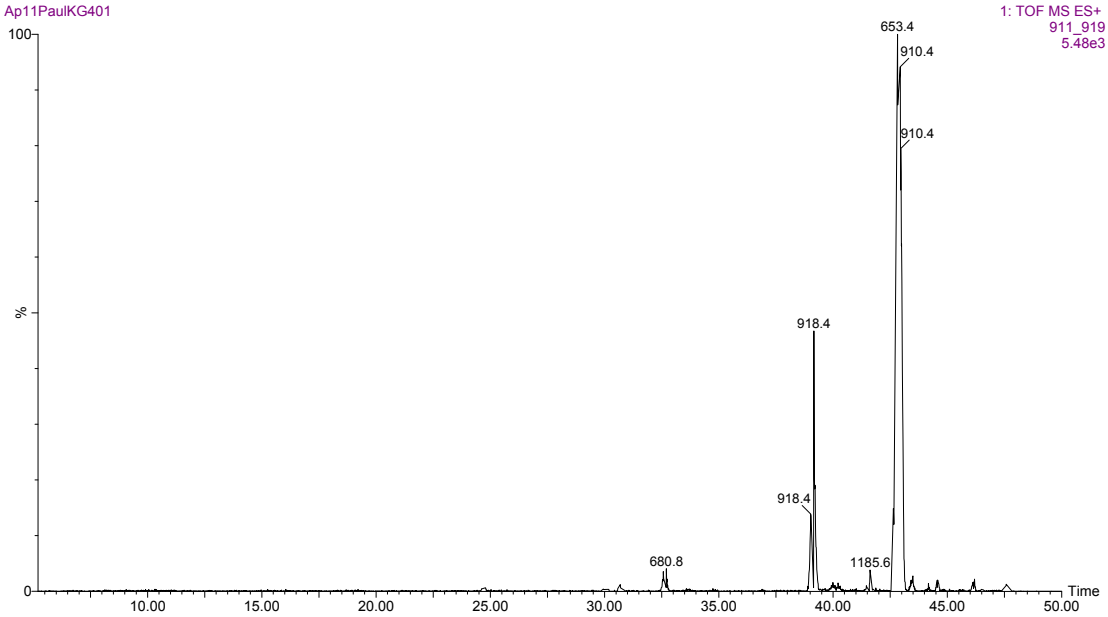
In order to verify the difference in composition of the protein subunits, corresponding size protein fragments were isolated for In-gel trypsin digestion. Expected and observed molecular sizes, amino acid sequences of peptide ions produced from excised 33, 31, 28, 26, and 21 kDa protein subunits with their matching proteins from Mascot search are presented in Table 4.4. Based on significant peaks of parent peptide ions obtained during "Quadrupole-Time of Flight -Electrospray ionization" (Q-TOF-ESI) analysis, three major proteins can be identified in different protein subunits. The 33 kDa protein is composed of ARL-2^{pa} protein subunits, 31 kDa subunit fragment also contains ARL-2^{pa} and arcelin (ARC^{pa}) proteins while the 28 kDa fragment is charged with PHA^{pa} and ARC^{Pa} protein subunits. The protein fragments from 26 kDa profile produced significant peaks for peptides that matched ARL-2^{pa} proteins only, while the 21 kDa subunit contained peptides for ARC^{pa} proteins (Table 4.4).

Table 4.4. Mascot search of observed, expected and calculated molecular sizes, amino acid sequences of peptide ions produced from excised 33, 31, 28, 26, and 21kDa protein subunits from G40199 and derived interspecific backcross hybrid are presented with their matching proteins from NCBInr database.

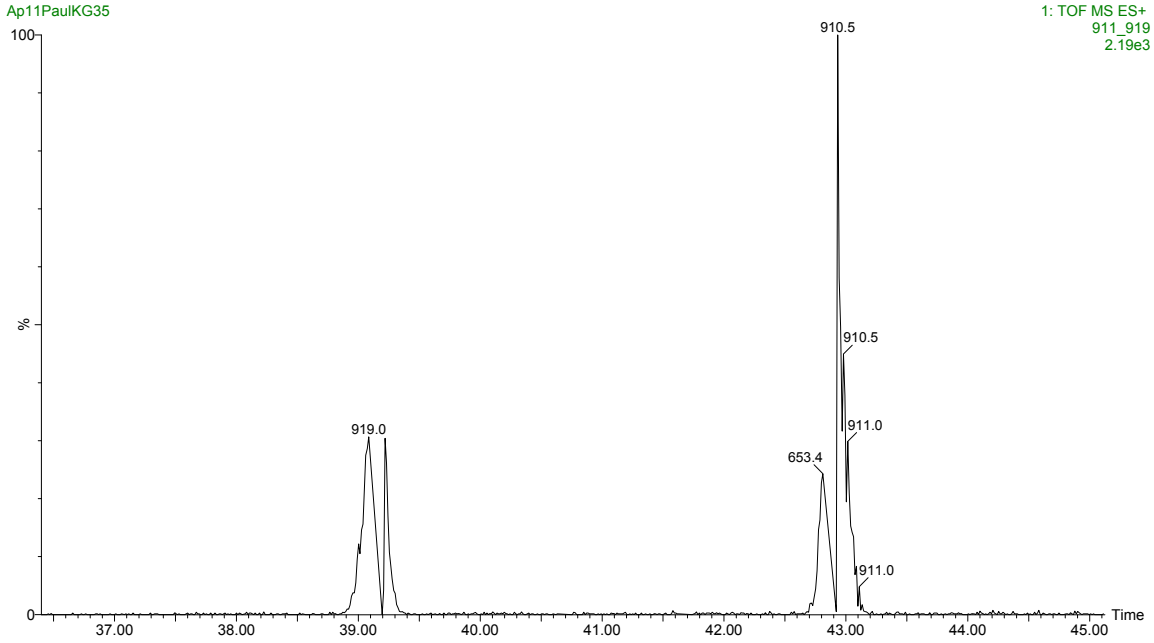
protein fragment	Observed	Mr. expt	Mr calc	Score	Peptide	Matched protein - reference
			----------G40199----------			
33kDa	910.41	1818.81	1818.82	110	R.LTGVGSNEDPWVDSMGR.A	ARL-2 [pa] - (NCBI AF255724)
	918.44	1834.87	1834.82	79	R.LTGVGSNEDPWVDSMGR.A + Oxidation (M)	ARL-2 [pa] - (NCBI AF255724)
			----------ICA Pijao x G40199 interspecific backcross----------			
33kDa	910.41	1818.81	1818.82	110	R.LTGVGSNEDPWVDSMGR.A	ARL-2 [pa] - (NCBI AF255724)
	918.46	1834.91	1834.82	58	R.LTGVGSNEDPWVDSMGR.A + Oxidation (M)	ARL-2 [pa] - (NCBI AF255724)
			----------G40199----------			
31kDa	910.40	1818.79	1818.82	118	R.LTGVGSNEDPWVDSMGR.A	ARL-2 [pa] - (NCBI AF255724)
	918.40	1834.79	1834.82	67	R.LTGVGSNEDPWVDSMGR.A + Oxidation (M)	ARL-2 [pa] - (NCBI AF255724)
	918.40	1834.79	1834.82	95	R.LTGVGSNEDPWVDSMGR.A + Oxidation (M)	ARL-2 [pa] - (NCBI AF255724)
	598.33	1194.64	1194.6	57	R.AFYSTPIQIR.D	ARC [pa] - Mirkov et al. 1994
	757.39	2269.15	2269.1	72	R.ANNAGHSAYGLAFALVPVGSEPK.R	ARC [pa] - Mirkov et al. 1994
28kDa	548.31	1094.61	1094.61	32	R.HIGIDVNSIK.S	PHA[pa] -Mirkov et al. 1994
	900.95	1799.88	1799.90	70	R.LTNLNDNGEPTLSSLGR.A	PHA[pa] -Mirkov et al. 1994
	997.53	1993.04	1993.06	47	R.VPNNAGPADGLAFALVPVGSK.P	PHA[pa] -Mirkov et al. 1994
	665.36	1993.07	1993.06	23	R.VPNNAGPADGLAFALVPVGSK.P	PHA[pa] -Mirkov et al. 1994
	740.41	2218.22	2218.21	69	R.VPNNAGPADGLAFALVPVGSKPK.D	PHA[pa] -Mirkov et al. 1994
	598.32	1194.63	1194.64	46	R.AFYSTPIQIR.D	ARC [pa] -Mirkov et al. 1994
	757.39	2269.16	2269.15	52	R.ANNAGHSAYGLAFALVPVGSEPK.R	ARC [pa] - Mirkov et al. 1994
26kDa	910.41	1818.81	1818.82	77	R.LTGVGSNEDPWVDSMGR.A	ARL2 [pa] - (NCBI AF255724)
	918.41	1834.80	1834.82	43	R.LTGVGSNEDPWVDSMGR.A + Oxidation (M)	ARL2 [pa] - (NCBI AF255724)
	918.41	1834.80	1834.82	8	R.LTGVGSNEDPWVDSMGR.A + Oxidation (M)	ARL2 [pa] - (NCBI AF255724)
21kDa	640.82	1279.63	1279.63	76	K.TDVQITYESPK.K	ARC [pa] - Mirkov et al. 1994

Score ≥ 50 indicates identity or extensive homology ($p < 0.05$).

Chromatograms from peptide sequencing of the 33 kDa fragment in G40199 and its derived interspecific backcross show similar peaks and sequence of parent ions (Fig. 4.14 a and b). The other protein peptide chromatograms from four protein profiles in G40199 produced significant peaks for representative peptides are presented in appendices 4.1, 4.2, 4.3, and 4.4.



(a)



(b)

Figure 4. 14 Chromatogram of m/z for protein peptide peaks from 33 kDa protein produced by Q-TOF- ESI (a) Accession G40199; (b) Interspecific backcross line ICA Pijao x G40199.

BLAST search of the resultant peptides sequence from 33 kDa and 31 kDa protein subunit of ions with molecular sizes of 909.9, 910.4 and 918.4 m/z from trypsin-digested peptides were identical to ARL-2^{pa} protein peptides with corresponding 19 amino acids residues that matched on ARL-2^{pa} protein of *P. acutifolius* spanning from amino acid 58 to 74 (Fig. 4.15). Similar peptides' amino acid sequences produced from a 33 kDa protein subunit were matched on the deduced amino acid sequences from cDNA sequences of ARL-3^{pa} gene from G40199 and Rojo interspecific backcross line.

```
  1 MASSNLLSRA LFLLLPTHAI SATDTYFNFD FFKQNDADTN RLILQRDATI
 51 SSGGRLRLTG VGSNEDPWVD SMGRAFYSDP IQIRDSTGNL ASFHTNFTFI
101 IRANNAGHSA YGLAFALFPV GSQPKRKREN LGLFPDAHTV AVFNTVSNVM
151 KSTSTPTRLA QRGFAISTNH NGETTDVQIT YESPKKNLKI VLPSTNSNVQ
201 YDFNAPLYLE NEVDRNVSVG FSATSGLTEE TTETHDILSW SFSSEFPDHT
251 TSEPSNILLN NIL
```

Figure 4. 15. Amino acid sequences from trypsin digested peptides of the ~33, 31 and 26 kDa protein bands from G40199 and interspecific backcrossed hybrids matched to the ARL2^{pa} complete amino acid sequence of *P. acutifolius.* Matching peptide sequences are shown in bold.

Other chromatograms of peptide peaks produced from the 31, 28 and 21 kDa protein subunits in G40199 with m/z of 598.3, 757.4 and 640.8 (Table 4.3) had amino acid sequences matching to arcelin (ARC^{pa}) of *P. acutifolius* (Fig. 4.16) and to a similar match to the deduced amino acid sequences from ARL-4^{pa} cDNA sequences of accession G40199 with minor amino acid sequence substitutions.

```
  1 MASSKLLSLA LFLLLLTHAT ATTFDFPTFH KEDKNRLILQ GNATISSGGR
 51 LRLTGVGSNE DPRVDSMGRA FYSTPIQIRD STGNLASFDN KFTFIIRANN
101 AGHSAYGLAF ALVPVGSEPK RKQEYLGLFP DAHTVVWCST PSATVLKSTS
151 TPTRLAQRGF AISTNTTEKT DVQITYESPK KNLRVVLHFT NSNVKYDFNA
201 PLYLENDVDR SVSVGFSATS GLKEETTETH DVLSWSFSSK FEPFYVKDQK
251 SEGSNILLNQ IL
```

Figure 4.16. Amino acid sequences from trypsin digested peptides of the ~ 31, 28 and 21 kDa protein bands from G40199 matched to ARC^{pa} complete amino acid sequence of *P. acutifolius*. Matching peptide sequences are shown in bold.

In addition to ARC^{pa} found in a 28 kDa protein subunit, significant peptide peaks were identified that matched to phytohaemagglutinin (PHA^{pa}) of *P. acutifolius*. BLAST search for homologous proteins using peptides' amino acid sequences produced matches three peptides with match to PHA^{pa} (Fig. 4.17). Peptide analysis from this band indicated that more than one lectin-like protein subunit was present, with largest score corresponding to PHA^{pa}.

```
  1 MASSNFSTVL SLALFLPLLT HANSANDISF NFQRFNETNL ILQGDASVSS
 51 SGQLRLTNLN DNGEPTLSSL GRAFYSTPIQ IWDSTTGAVA SFATSFTFNI
101 RVPNNAGPAD GLAFALVPVG SKPKDRGGLL GLFDGSDSRA HTVAVEFDTL
151 YNRDWDPRER HIGIDVNSIK SIKTTPWDFG QGEDAEVLIT YDSSTKLLVA
201 SLVYPSQKTS FIVSDTVDLK SVLPEWVRVG FSATSGITKG NVETNDLLSW
251 SFASKLSDGT TSEGLNLANF VLNQIL
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Figure 4.17. Amino acid sequences of trypsin digested peptides on the ~ 28 kDa protein band from G40199 matched on the phytohaemagglutinin complete amino acid sequences from *P. acutifolius*. Matching peptide sequences are shown in bold.

DISCUSSION

cDNA sequencing and phylogenetic analysis

Total RNA extraction and development of cDNAs for ARL-3^{pa} and ARL-4^{pa} genes from accession G40199 demonstrated the expression of novel variants of arcelin in this accession. A complete genomic DNA sequence of ARL-2^{pa} was previously described and deposited in NCBI accession AF255724 from an unidentified genotype of tepary bean accession. Arcelins do not contain introns; therefore the same primers amplified identical fragments from genomic DNA and expressed cDNA. ARL-3^{pa} was expressed in conjunction with ARL-4^{pa} a second arcelin protein that is related to an arcelin variant in tepary bean reported by (Mirkov et al., 1994). In the previous study, ARC^{pa} was not detected in genomic DNA of accession G40199 and interspecific hybrids using ARC^{pa} primers. But when the same ARC^{pa} primers were used for PCR amplification of cDNA from accession G40199 and interspecific hybrids, a fragment of approximate 800 bp was amplified. The amplification of a second arcelin variant in the G40199 agrees with the finding of protein subunits for arcelin and ARL-2^{pa} related proteins in peptide analysis. The inability to amplify this gene (ARC^{pa}) from genomic DNA suggests that even though arcelins are said to be intron-less genes, some post-transcription editing may occur probably as RNA editing thus may require designing of new gene specific primers. The finding of two different arcelins expressed in a homozygous condition in this accession was unexpected and to our knowledge, has not been reported previously. Because APA is known to be a complex locus consisting of tandem duplications that have then undergone divergent evolution (Lioi et al. 2003; 2006), it is possible that ARL-3^{pa} and ARL-4^{pa} are

tightly linked loci. This also raises the question of whether the other arcelins known in *P. vulgaris* are actually alleles of a locus, or are tightly linked tandem loci that give the appearance of alleles at a single locus. If the latter were true, then it might be possible to achieve recombination among arcelin genes to bring them into coupling. Such an "arcelin cassette" would be of great value in breeding for bruchid resistance because multiple arcelins combined may produce higher and broader spectrum resistance. We have developed in this work a population of backcross lines expressing *arcelin*-2 (*Arl-2*^{pv}) from *P. vulgaris* and the ARLs from G40199 in a phaseolin null background. Preliminary bruchid screening of these materials indicated a high degree of resistance to *A. obtectus* in a homozygous and heterozygous state (chapter 5 Appendix 5.8).

We used a cultivated *P. acutifolius* accession (Brown Tepary) in this work to determine the genomic difference on the composition of LLPs between cultivated and a wild species. Interspecific hybrids developed by introgression of the LLPs from G40199 were used to confirm the stable introgression of the ARL genes into common bean interspecific backcross lines. The expression of LLPs in interspecific hybrids behaved in the same way as in the parent G40199 with minor amino acid sequence modifications a common trend of lectin proteins as observed in phylogenetic analysis. Similar characteristic gaps for arcelin proteins demonstrating their evolutionary trend from parental lectins were observed in tepary bean arcelins and common bean arcelins. However, tepary bean arcelin variants from both cultivated and wild types demonstrated additional gaps and amino acid sequence substitutions indicating higher genetic difference from their relative arcelins of *P. vulgaris* (Fig. 4.12). These differences may be associated with functional properties of tepary bean arcelins. The presence of specific

gaps in tepary bean arcelin may also be related to proteolytic processing that might have led to the production of four main arcelin protein subunits observed in protein gels and peptide sequencing. It is unclear how arcelin protein subunits are made or if the gaps are responsible as protein processing sites to make a different subunit of a functional protein. This may require detailed understanding of the protein chemistry of the lectin-like seed storage proteins that was beyond the scope of this work. Amino acid substitutions can be related to the formation of a new variant or alteration of the protein activity in a given genotype.

The arcelin variant found in Brown Tepary (ARC^{pa}-BT) is similar to the one reported by Mirkov et al. (1994) apart from minor amino acid substitutions that are probably genotype specific. The identity of the accession used to develop DNA sequences by Mirkov et al. (1994) was not specified. They used a cultivated accession of *P. acutifolius* and the wild accession G40102, and it may be possible that Brown Tepary used in the current work is similar to the cultivated accession used by Mirkov et al. (1994). The LLPs reported by Mirkov et al. (1994) were not different between those in cultivated accession *P. acutifolius* and G40102 used in their work because only one sequence was reported from the two accessions used. Our work has demonstrated a difference in LLP composition and variants between cultivated Brown Tepary and wild accession G40199. ARL-3^{pa} has high sequence identity to ARL-2^{pa} of the unknown accession of *P. acutifolius* (NCBI- AF255724) and is only present in wild accession G40199 and its derived interspecific hybrids but absent in cultivated accession of Brown Tepary.

The second arcelin variant ARL-4^{pa} found in G40199 and its hybrids as cDNA demonstrates close nucleotide sequence identity to mRNA of ARC^{pa}, and has 72% amino acid identity. This is relatively low amino sequence identity with the present LLPs published in tepary bean, and may represent evolution of a new arcelin variant. The amino acid alignment in a phylogenetic analysis demonstrates that it is a separate variant from the other arcelins in *P. acutifolius* and shows its intermediate relationship to *Arl-5* of *P. vulgaris*. The closer genetic relationship of some arcelins from *P. acutifolius* to arcelin 5 of *P. vulgaris* is consistent with phylogenetic relationship demonstrated by Lee et al. (2002). This novel variant also demonstrated extensive amino acid substitutions that separate it from the cluster of other tepary arcelins. Full sequence of this variant may be reconstituted by cloning and performing 5'-RACE (Schramm et al. 2000). Sequence developed in this work is from a direct PCR product of isolated single cDNA fragment using primers designed to span the maximum coverage of the gene sequence. The expressed cDNA for ARL-3^{pa} gene in G40199 and its interspecific hybrids demonstrated high sequence identity to the ARL-2^{pa} (NCBI AF255724) with minor differences.

The cDNA sequences developed from accession G40199 have useful genomic information for exploiting the two genes in G40199 for marker assisted selection of bruchid resistance, and for further isolation of the full gene sequence for genetic transformation for insect resistance in related crop species. The partial ARL sequences developed from this accession will be deposited into a database for public use.

We could not obtain cDNA products for expression of α-AI^{pa} in any of the genotypes used in this work although the gene was present in all genomic DNA samples as detected by amplification with α-AI^{pa} gene specific primers. It should also be noted

that no α-AI^{pa} or PHA were amplified in cDNA samples not treated with RT-transcriptase, indicating that the other amplified cDNA products were not accidental genomic DNA contamination. The absence of expression of these two genes in all samples was not expected knowing lectin-like genes are intron-less. Several possible explanations for this may be suggested. The evolutionary nature of lectins and tandem duplication activity of genes in the complex APA locus may be a factor that leads to selective expression of one or more LLP variants in legumes (Sparvoli et al. 1998; Lioi et al. 1999; 2003; 2006). Alternatively it may be possible that genomic DNA for α-AI^{pa} in G40199 and interspecific hybrids is transcribed and translated to produce the second arcelin variant observe only in cDNA but was not obvious in genomic DNA. The alternative mRNA processing or editing of α-AI^{pa} gene leading to a new arcelin variant might be a mechanism for the plant to produce increased arcelin concentration in the seed for enhanced antibiosis.

Since α-AIs specifically protect against starch digestion in the seeds, and G40199 seeds are small and may contain less carbohydrate, then α-AI may not necessarily accumulate at an early stage of seed development, but is likely to accumulate later during seed maturation. Thus the stage at which total RNA was isolated may not contain appreciable expression of the protein. It is possible that the lectins in tepary beans undergo selective mRNA editing/splicing (Cheah et al. 2007) and proteolytic processing before the final synthesis of active protein. Thus the removal of C-terminus sequences is likely to affect the specific PCR amplification of the unique nucleotide sequences from the cDNA for alpha amylase inhibitors. This may result in a premature drop of both forward and reverse primers due to a lack of annealing of complementary nucleotide

sequences for the consecutive PCR cycles. Different primer design may be required to target the edited mRNA (cDNA) for α-AI^{pa} and PHA^{pa} as a result of nucleotide substitutions or deletions during RNA processing if these genes contain introns, a situation to be validated. An alternative solution to demonstrate the expression of the entire APA locus of genes was to analyze the lectin-like protein composition by peptide sequencing of LLP fragments obtained from the SDS-PAGE on total storage seed proteins.

Protein peptide sequencing

Peptide sequencing provided evidence of the expression of arcelins (ARL) and PHA, but unfortunately we could not determine any peptides with amino acid sequences for α-AI proteins of tepary bean. Similar difficulties were encountered in determining the expression of α-AI in cDNA. A protein fragment of approximately 21 kDa was isolated from G40199 in prospect that was a possible candidate for the location of α-AI protein subunits from total seed storage protein profiles. However, this fraction contained peptides of arcelin variant (ARC^{pa}). Yamada et al. (2001, 2005) identified α-AI^{pa} protein subunits using antibodies at a molecular size of 15-18 kDa. The estimated molecular size on SDS gel was probably higher and we unfortunately could not isolate the weakly expressed lower molecular size (14 -19 kDa) protein subunits from the total seed storage proteins for In-gel peptide sequencing to confirm this. For α-AI^{pa}, if mRNA is cleaved into lower molecular weight subunits (a common process in LLPs that leads to C-terminus truncation of mature proteins), it is possible that α-AI^{pa} was converted to other isolectins (Young et al. 1995). This process could make it possible for conversion of α-

AI into an arcelin isoform. In contrast to arcelin and PHA, α-AI is synthesized as a glycosylated precursor of about 40kDa, which is proteolytically processed in the protein storage vacuoles into 15-20 kDa polypeptides of the mature inhibitor (Pueyo et al. 1993; Santino et al. 1992).

Considering the resistance to proteolytic degradation of LLP proteins, it was not possible to observe several LLP peptides in all of the protein profiles studied when compared to the theoretically predicted peptide residues that can be generated from trypsin digestion. Most of the proteins that are associated with resistance to bruchids are likely to be highly resistant to proteolytic digestion by enzymes like trypsin, chymotrypsin and pepsin. Therefore it may be impossible or rare to observe a highly representative number of N-terminal cleavages of peptides from such proteins. In this case one may think that those proteins are absent in the total protein extract from mature bean seeds.

Besides the difficulties in detecting α-AI proteins, peptide isolation and sequencing has clearly demonstrated that the APA locus present in accession G40199 is a complex locus that is processed into variants of active arcelin proteins in the mature seeds for protecting seeds from predation. It will be important to determine the protein composition of lower molecular weight proteins using the same procedure. Increasing the amount of total protein samples for SDS-PAGE may produce a higher quantity of proteins in the lower profiles. This may facilitate the isolation of lower molecular size protease inhibitors such as trypsin inhibitors that are common components in tepary bean (Campos et al. 1997, 2004). We have recently observed a larger molecular sized protein

profile that behaves as a subunit of the major 33 kDa protein since it co-segregates in the interspecific hybrids. Verification of the identity of this protein is required.

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CHAPTER 5

Evaluation of A. *obtectus* resistance among interspecific hybrid backcross families segregating for APA proteins from tepary bean accession G40199.

Kusolwa PM and Myers JR

Abstract

Wild tepary beans (*P. acutifolius*) contain arcelins and arcelin-like proteins that are co-expressed in the arcelin - phytohaemagglutinin - α-amylase inhibitor (APA) locus. Two arcelin variants ARL-3^{pa} and ARL-4^{pa} proteins were identified in a wild tepary bean accession G40199. The genes were transferred into common bean cultivars ICA Pijao and Rojo by interspecific hybridization via embryo rescue and bridge parent hybridization. Inbred backcross lines were developed and selected for segregation of the protein at BC_2F_3 generations. Seeds from homozygous and heterozygous lines expressing the two arcelins and introgression lines lacking the proteins were used to evaluate the effect of the arcelins on *A. obtectus* reproduction and growth. Identification of arcelin containing lines was based on the co-segregating 33 kDa lectin-like seeds storage protein inherited from tepary bean G40199. Significant mean delay of 63 days for adult emergence and low number of emerged F_1 *A. obtectus* adults were observed in lines with homozygous expression of ARL-3^{pa} and ARL-4^{pa} (designated ARL proteins in this paper). Adult insect size from ARL-containing introgression lines was reduced and weight was halved. While not as high as the G40199 parent, levels of resistance in the introgression lines appear to be economically useful.

INTRODUCTION

In the tropics, dry bean production is constrained by several biotic and abiotic factors. Some of the major biotic constraints involve post-harvest losses caused by two major bruchid species *A. obtectus* and *Z. subfasciatus*. The bean weevil, *A. obtectus* is a predominant post harvest pest in all tropical highland bean producing regions attacking beans in the field and in storage. The pest causes quality degradation and poor germination of damaged seeds.

In an effort to find sources of resistance to these pests, seed proteins of the lectin family called arcelins were discovered in wild common bean accessions (Osborn et al. 1988) and were associated with bruchid resistance. Highest levels of resistance to *Z. subfasciatus* was conditioned by *Arcelin-5* (*Arl-5*) followed by *Arl-1*, *Arl-2* and *Arl-4*, while *Arl-3* exhibited moderate resistance (Cardona et al. 1990; Kornegay et al. 1993). *Arl-2,- 4*, and *-5* provide moderate resistance to *A. obtectus* (Kornegay and Cardona 1991; Goossens et al. 2000). Some arcelin alleles have been backcrossed into cultivated dry bean to develop bruchid resistant cultivars (Osborn et al. 1986; 1988; Cardona et al. 1990; Kornegay and Cardona 1991; Kornegay et al. 1993; Misangu 1997).

In evaluating the insecticidal efficacy of *Arl-5*, Goossens et al. (2000) demonstrated that arcelin was not the only factor for resistance to the two weevil species, concluding that factors linked to arcelin may be responsible for resistance. On the other hand, other arcelin alleles confer high levels of resistance without attenuation during the transfer from wild to cultivated common bean. This is the case with *Arl-1* transferred into improved Tanzanian dry bean cultivars to provide resistance to *Z. subfasciatus* (Misangu, 1997; Myers et al. 2001). Bruchid resistance can also be increased by

combining arcelin with a phaseolin null gene. A greater proportion of the seed storage protein is expressed as arcelin to compensate for the loss of phaseolin, thereby increasing the toxicity of the seed to bruchids (Hartweck et al. 1997).

High levels of resistance to two major bruchid species was demonstrated in tepary bean by Shade et al. (1987) who recommended them as potential sources of resistance. Pratt et al. (1984, 1990) reported that lectins present in some tepary bean accessions conferred high levels of resistance to *A. obtectus*. Arcelin and arcelin-like proteins have been reported in cultivated accessions of tepary bean *P. acutifolius* (Mirkov et al. 1994; NCBI -AF255724) but their resistance to *A. obtectus* was not characterized. It is likely that arcelin and arcelin-like proteins of tepary bean exist as different alleles and variants (Lioi et al. 2003, 2006). Previously, researchers at CIAT had reported that the accession G40199 conferred high levels of resistance to both bruchid species (Mejia-Jiménez et al. 2002; Cardona et al. 2005). We found that this accession contains a complete arcelin-phytohaemagglutinin and alpha amylase inhibitor (APA) locus of the lectin-like proteins (LLP) with expression of two arcelin variants ARL-3^{pa} and ARL-4^{pa} referred here as ARL proteins. The two proteins show similarity to ARL-2^{pa}, and an arcelin previously described in NCBI database accession AF255724 and by Mirkov et al. (1994) respectively. G40199 also contains phytohaemagglutinin and alpha amylase inhibitors (α-AI^{pa}), whose isoforms were only specific to those in *P. acutifolius* variants (Chapter 3 & 4). We hypothesized that the simultaneous transfer of these proteins into common bean cultivars would improve resistance to *A. obtectus* and perhaps *Z. subfasciatus* as well. These proteins were transferred into common bean by interspecific hybridization (Chapter 3), and the desired protein expression in interspecific hybrid progeny was

confirmed. The objective of the present research is to evaluate the association of the introgressed APA locus from G40199 with bruchid resistance.

MATERIALS AND METHODS

Plant materials and genotype identification

Three interspecific hybrid backcross families were used to screen for *A. obtectus* resistance. A BC_2F_3 family from the cross between ICA Pijao x G40199 included 93 lines, a second family had 33 BC_1F_3 lines from the cross between the interspecific bridge parent and Rojo, an improved large red seeded cultivar from the Sokoine University of Agriculture bean breeding program in Morogoro, Tanzania. A third family consisted of 44 backcross lines of the cross between interspecific bridge parent (ICA Pijao xG40199) x (*Arl-2*^{pv} with phaseolin null Rojo backcross line) and was segregating for ARL proteins and phaseolin. The *Arl-2*^{pv} with phaseolin null lines were developed from a parallel breeding program to introgress *Arl-2*^{pv}-phaseolin null from SMARC lines (Hartweck et al. 1997) into Rojo cultivar. Throughout the remainder of the chapter, interspecific hybrid backcross materials are referred to as introgression lines or families.

Eleven single plants from each introgression family were randomly selected for genomic DNA analysis for the presence of ARL-3^{pa}, α-AI^{pa} and PHA (described in Chapters 3 & 4). It was not possible to separate homozygous and heterozygous plants, therefore, seeds from ARL-3^{pa} + single plant selection (SPS) were progeny tested in the BC_2F_3 by protein gel electrophoresis to identify individual lines that did not segregate for the 33 kDa protein associated with PHA^{pa}, ARL-3^{pa} and ARL-4^{pa}. Total seed proteins were analyzed on eleven individual seeds from each individual backcross line using 15% tris-HCl SDS-PAGE as described in chapter 3. Three genotypes were identified: homozygous (ARL/ARL - 26 lines), heterozygous (ARL/arl - 64 lines) and homozygous null (arl/arl - 36 lines) for the 33 kDa profile associated with ARL proteins (Appendix

5.9). Four samples containing bean seeds from ‘Rojo’, and ‘ICA Pijao’ were used as susceptible checks while G40199 seeds were included as resistant control.

Screening for resistance to *A. obtectus* among interspecific hybrids

A spontaneously appearing colony of *A. obtectus* was obtained from bean stocks in the Oregon State University (OSU) snap and dry bean breeding program. The bruchid ecotype was identified based on specific characteristic features such as species-specific orange-colored last segment of the antennae that corresponded to *A. obtectus* (Gonzalez-Rodriguez et al 2000). The colony was multiplied and maintained in susceptible Rojo and Pinto (cultivar unknown) bean seeds at OSU to obtain enough adult insects for inoculation of experimental materials. Due to a limited size of the bruchid colony, inoculation of *A. obtectus* adults was conducted at different times for each backcross population of interspecific hybrids.

Fifteen non sexed *A. obtectus* adults were placed into glass vials containing 30 seeds in the case of ICA Pijao introgression population and 20 seeds for the Rojo backcross population. Vials were loosely closed to allow aeration but prevent escape of adult insects. Glass vials containing seeds and adult insects were placed into incubation trays and kept undisturbed for 12 days at 25 ± 3°C, and ambient relative humidity during the fall, winter and spring of 2006/2007. After 12 days, number of eggs laid was estimated using a magnifying lens, and adults were removed. In cases where no eggs were visible, samples were re-inoculated with new adults. Vials were placed back into the incubation tray and left undisturbed but monitored until the first adult emerged. Powdery/floury appearance on the surface of the beans was noted to be sure of the larvae penetration into the seeds and to identify possible escapes. Following the observation of

the first emergence of F_1 adults, each vial was inspected daily and emerging F_1 adults were removed to avoid any new egg laying. Emerging F_1 adults from each sample were counted daily up to 72 days after initial inoculation. Further observation to 100 days was continued in some lines that showed delayed emergence, especially in samples of G40199 tepary beans and some interspecific hybrid lines that demonstrated long delays. Furthermore, in an attempt to better understand the mechanisms of resistance in G40199, the testa was removed before feeding trials. This was to ensure easy entry of the larvae into the seeds and determine whether seed composition or hard seed coat was the major factor that completely inhibited adult emergence.

Data were collected for the total number of F_1 adults emerging after inoculation, number of days for first adult emergence (DAE), number of days for 50% of total F_1 adults emerged and susceptibility index (SI) calculated as:

$$SI = \frac{\text{Log}(\#\text{Adult emerged})}{\text{DAE}} \text{X}100$$

Other variables collected were; the number of perforated seeds, severity of damage expressed by the number of seeds with 5 or more holes and percent seed weight loss.

Frequency of adult emergence was determined by the total number of F_1 adults emerged per day for the period of 72 days. During the experiment, 54 samples of several emerging adults were collected from vials of beans with and without the ARL proteins and weight of 10 adults taken for each group.

Variables were subjected to statistical analysis using SAS 9.1.3 and S-PLUS 6.2. Analysis of variance (PROC GLM) with estimated number of eggs as a covariate was

used to adjust dependent variables for different number of eggs laid in different vials, and regression analysis was used to determine the association of novel seed proteins with *A. obtectus* resistance.

RESULTS

Effect of APA locus proteins on *A. obtectus* growth and reproduction

The effect of differing genotypes for ARL proteins on six variables observed during the *A. obtectus* feeding trial is presented in Table 5.1. G40199 by comparison completely inhibited adult emergence and did not affect seed parameters. The susceptible check behaved in a similar response as interspecific backcross lines lacking the ARL protein from G40199. Interspecific hybrids with homozygous expression of the ARL protein demonstrated positive response to variables related for resistance to *A. obtectus* in the two different interspecific introgression lines (ICA Pijao and Rojo Backcross families).

Table 5.1. Means[z] for variables from genotypes of interspecific hybrids' seeds segregating for the expression of a 33 kDa ARL proteins subjected to *A. obtectus* feeding trial, N = 26 for ARL/ARL, 64 for ARL/arl and 36 for arl/arl genotype.

Genotype		Bruchid F_1		Seeds			
		50% F_1 emergence (Days)	Emerged (#)	Perforated (%)	Severity >5 holes (#)	SI[y]	Weight loss (%)
G40199		∞	0	0	0	0	0
Rojo		45^{C}	109^{A}	80^{A}	4.8^{A}	4.6^{A}	21^{A}
arl/arl	RojoBC	47^{C}	51^{B}	43^{B}	5.0^{A}	3.6^{A}	17^{A}
ARL/arl	RojoBC	52^{B}	33^{C}	38^{C}	2.9^{B}	2.5^{B}	10^{B}
ARL/ARL	RojoBC	62.5^{A}	24^{D}	$28^{D\dagger}$	2.1^{C}	1.8^{C}	9^{B}
ICA-Pijao		44^{C}	57^{A}	52^{A}	4.3^{A}	3.9^{A}	23^{A}
arl/arl	ICAP BC	46^{C}	64^{A}	48^{A}	3.9^{A}	3.7^{A}	18^{B}
ARL/arl	ICA-P BC	53^{B}	36^{B}	29^{B}	2.7^{B}	2.7^{B}	8^{C}
ARL/ARL	ICAP BC	63^{A}	18^{C}	26^{BC}	1.7^{C}	1.6^{C}	8^{C}

[z]Means based on 20 seeds per vial for BC-Rojo and 30 seeds per vial for BC-ICA Pijao respectively. Means with the same letter in each BC population are not significantly different (Fisher's LSD of means) at $\alpha = 0.05$. [y]Susceptibility index.

i. *Days for 50% adult F_1 emergence*

Significant delay for 50% F_1 adult emergence was observed for introgression lines containing the tepary bean ARL proteins in either homozygous or heterozygous condition (Prob. <0.0001 α =0.05, Appendix 5.1). Adult bruchids emerged from families without ARL proteins in 46 days after inoculation. While the average of 53 days elapsed before 50% F_1 adults emerged in heterozygous lines and 63 days after inoculation for homozygous ARL lines. Regression analysis indicated 58% of the phenotypic variation in delayed number of days for adult emergence was explained by the ARL genotypes segregating for the presence of 33 kDa protein (Fig. 5.1a).

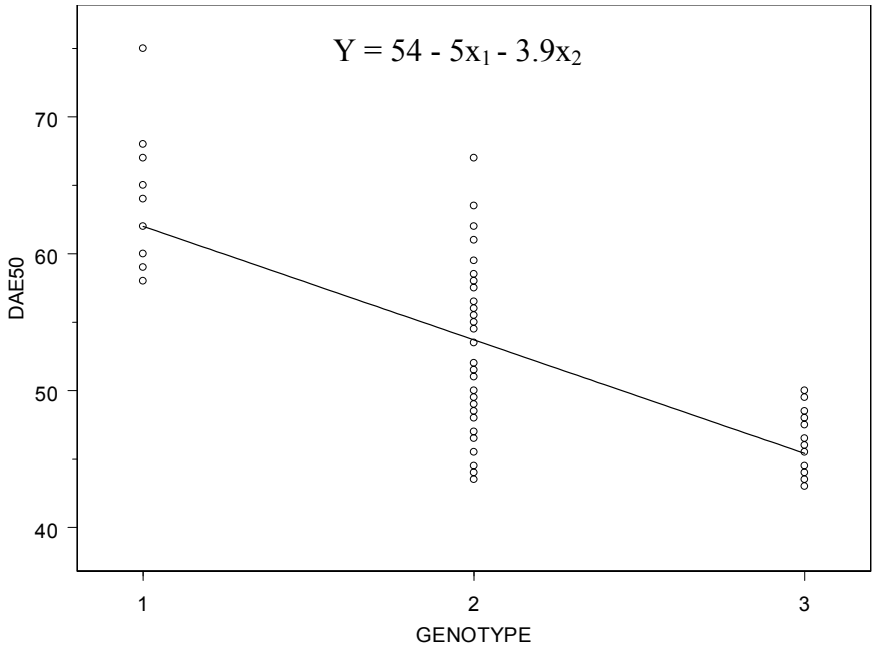

Figure 5.1a: Relationship between days for 50% F_1 adult bruchid emergence (DAE50) and performance of ICA Pijao backcross genotypes segregating for the 33 kDa proteins. Genotypes 1 = ARL/ARL, 2 = ARL/arl, 3= arl/arl.

ii. *Reduction of total number of emerging F_1 adults*

Total F_1 adult emergence was significantly different among the families of introgression genotypes Prob. < 0.0001 $\alpha = 0.05$ (Appendix 5.2). The lowest number of emerging F_1 adults was observed in genotypes that were homozygous for the expression of ARL proteins (Table 5.1) with only an average of 18 F_1 bruchid adults per line emerging in a 72 day period, compared to 64 mean adult emergence in lines lacking the proteins. Seed lots heterozygous for ARL demonstrated intermediate numbers of emerging adults. The highest number of emerging adults was seen in seed lots that did not express the ARL proteins. In ARL containing lines with the lowest emergence, most of late emerging adults were small in size.

As is obvious from table 5.1, reduction in number of emerging adults and reduced frequency of F_1 adult emergence are associated with seeds containing the ARL proteins. A slow rate of adult emergence was observed in introgression lines that were homozygous for the ARL proteins with some adults emerging as late as 80 to 90 days after inoculation. A proportion of late emerging adults were also observed in segregating families for 33 kDa protein probably demonstrating the dosage effect of the ARL proteins. In lines lacking the 33 kDa protein associated with ARL proteins, emergence was early with a sharp peak and occurred over a shorter time period (Fig. 5.1b). In homozygous ARL containing seeds, slow rate of emergence signals a significant retardation of the reproduction cycle and reduced fitness for the insect attempting to subsist and breed on the ARL containing beans. In Figure 5.1b, the peaks correspond to the mean number of days for 50% of F_1 adult emerged in each of the segregating ARL genotypes.

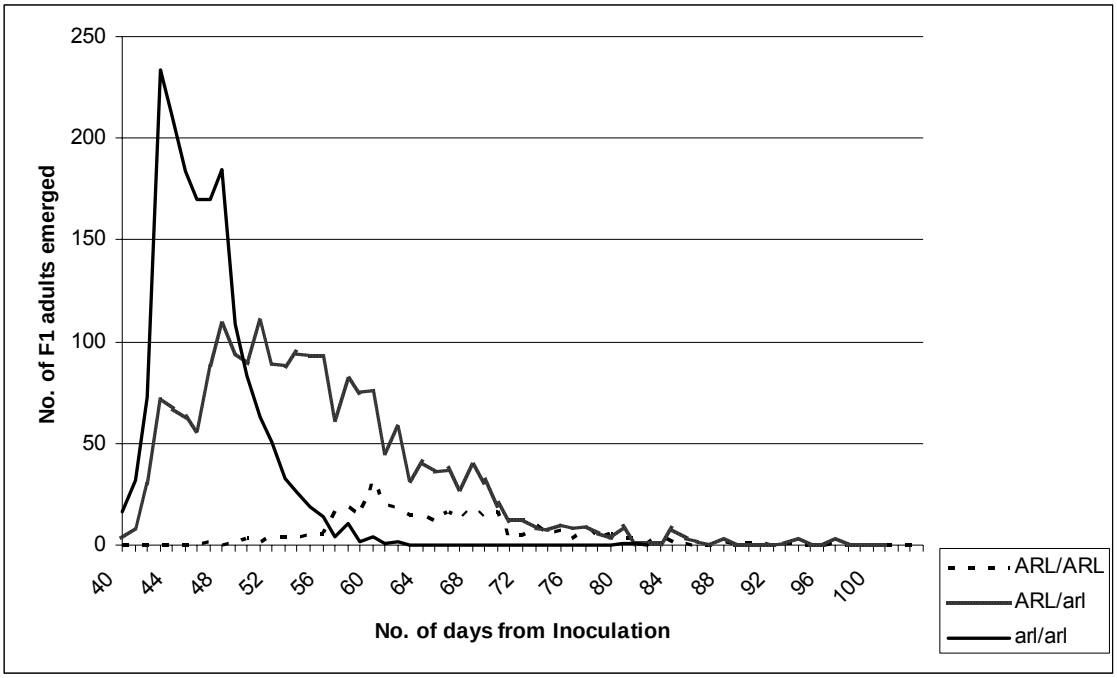

Figure 5. 1b Frequency of emergence of F_1 *A. obtectus* adults in interspecific hybrid backcross lines of ICA Pijao x G40199 with distinct genotypes for ARL seed storage proteins from tepary bean accession G40199. ARL/ARL are homozygous lines for the 33 kDa seed protein indicated by broken lines, ARL/arl are the segregating lines for the protein shown in shaded line, while arl/arl are the homozygous lines without the 33 kDa proteins shown in solid line.

iii. *Reduction of seed damage*

Seed damage expressed as percentage perforated seeds in the sample and severity of perforation determined by the number of seeds with five or more holes was analyzed. The presence of ARL proteins was associated with significant differences in percentage of seeds perforated/damaged (Prob. < 0.0001 $\alpha = 0.05$ (Appendix 5.3 and 5.4) as a result of larval feeding and emergence. Beans seeds showed 19 and 22% reduction of seed damage, respectively for heterozygous and homozygous introgression lines (Table 5.1). Homozygous and heterozygous lines did not show significant differences in percent damage, and very few seeds with 5 or more holes were observed in seed lots with either homozygous or heterozygous for ARL. There were two- to three-fold more seeds with

greater than five holes in lines lacking the 33 kDa tepary bean proteins compared to either ARL-homozygous or heterozygous lines (Table 5.1).

iv. *Susceptibility index and relation to seed damage*

Susceptibility index (SI) expressed as the logarithm of the number of F_1 adult emerged by the number of days for 50% F_1 adult emergence was significantly different among ARL containing genotypes (Prob. < 0.0001 $\alpha = 0.05$ Appendix 5.5). The lowest SI value of 1.6 was obtained in lines homozygous for ARL, heterozygous lines were intermediate, and homozygous lines lacking the arcelins had the highest mean SI of 3.7 (Table 5.1). The lower susceptibility index illustrated the ability of the introgressed proteins to reduce the number and rate of adult emergence. SI was negatively correlated to number of days for adult emergence (Fig. 5.2).

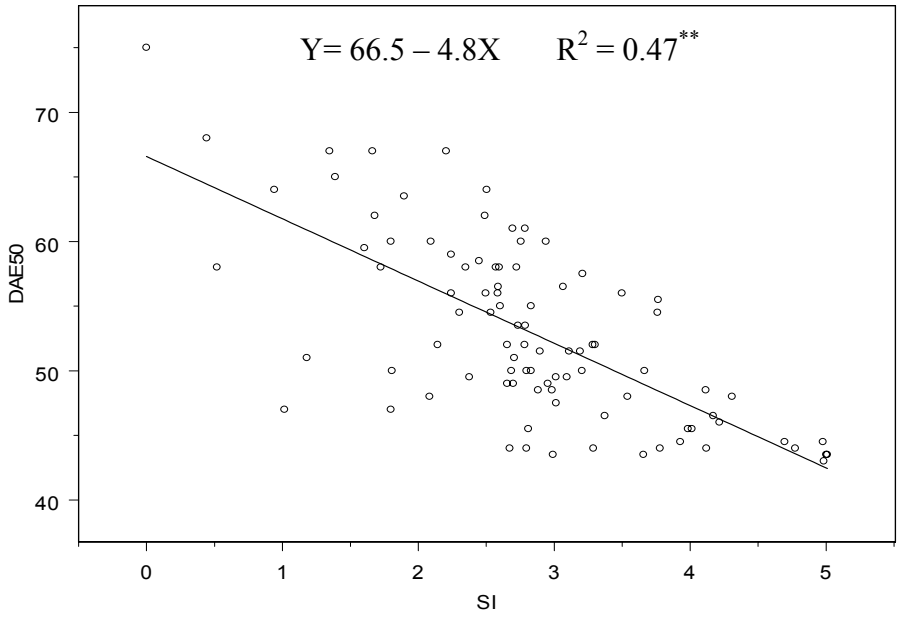

Figure 5. 2. Relationship between days for 50% F_1 adult *A. obtectus* emergence (DAE50) and susceptibility index (SI) in ICA Pijao common bean interspecific backcrossed lines containing novel seed storage proteins from G40199 tepary bean.

Susceptibility index (SI) also demonstrated an exponential relationship with number of emerging adults indicating that susceptible seeds with high SI had exponentially more adult emergence (Fig. 5.3). The presence of arcelin contributed to significant decrease in total number of emerging F_1 adults in a given time of storage. This reduced the rate of seed damage for bean stocks containing the protein.

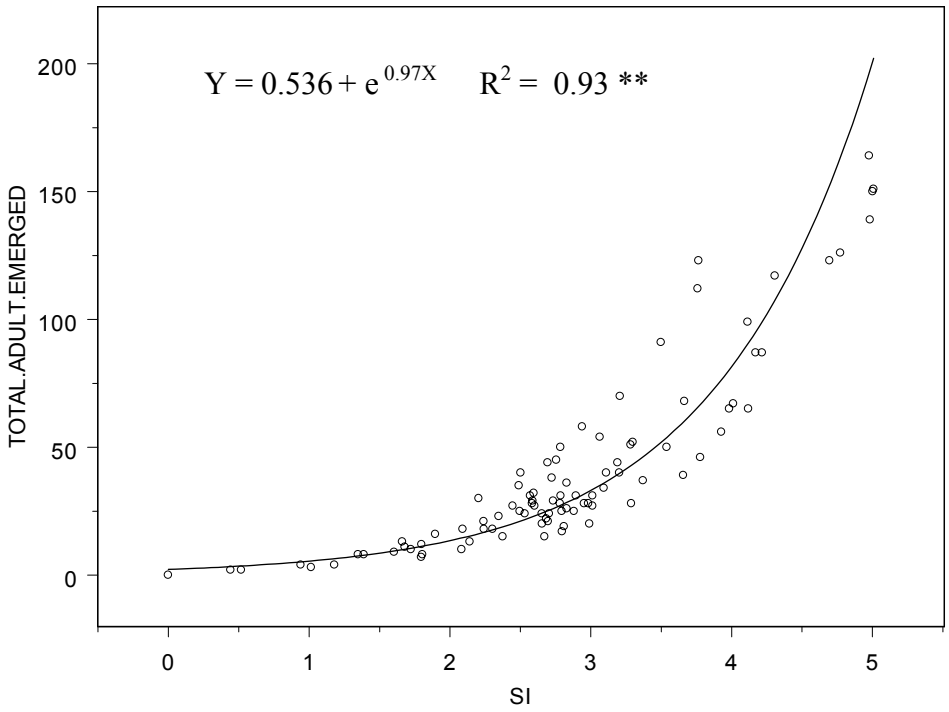

Figure 5.3. Relationship between total number of emerging F_1 bruchid adults and susceptibility index (SI) in ICA Pijao common bean interspecific backcrossed lines containing novel seed storage proteins from G40199 tepary bean.

v. *Reduction of seed weight loss*

Significant reduction in seed weight was observed as a result of larvae feeding on the seeds (Prob.= 0.0014 $\alpha = 0.05$; Appendix 5.6). Families containing arcelin had lowest mean seed weight loss of 8 % compared to a mean seed weight loss of 18% in families lacking the protein (Table 5.1). Low seed weight loss in arcelin containing seeds might

stem from fewer larvae infesting a seed as well as less feeding by larvae inside the seed. Seeds from segregating genotypes were not significantly different from homozygous ARL containing seeds.

Seed weight loss was highly correlated with severity of damage. Bean seed lots with severe perforation (5 or more holes/seed) demonstrated a high percent seed weight loss (Fig. 5.4). Bean samples without ARL proteins had the highest number of severely damaged seeds with 5 or more holes and suffered a higher percent seed weight loss. Seed weight loss was also highly correlated to total number of perforated seeds.

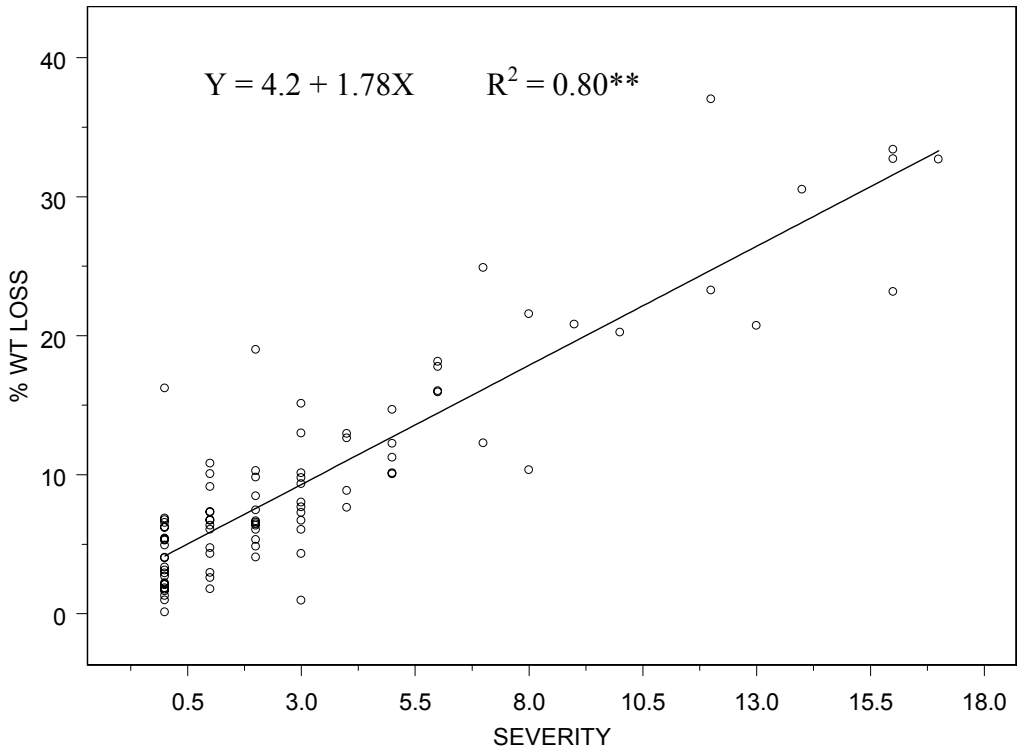

Figure 5.4. Relationship between percent seed weight loss (% WT LOSS) and severity of damage (number of seeds with > 5 holes) (SEVERITY) after *A. obtectus* damage to ICA Pijao common bean introgression lines containing novel seed storage proteins from G40199 tepary bean.

On the other hand, percent seed weight loss had a quadratic relationship with the number of emerged F_1 adults (Fig. 5.5). Initially seed weight loss increased quickly followed by a gradual increase at a point where the number of emerged adults becomes large indicating competition for resources from the seed.

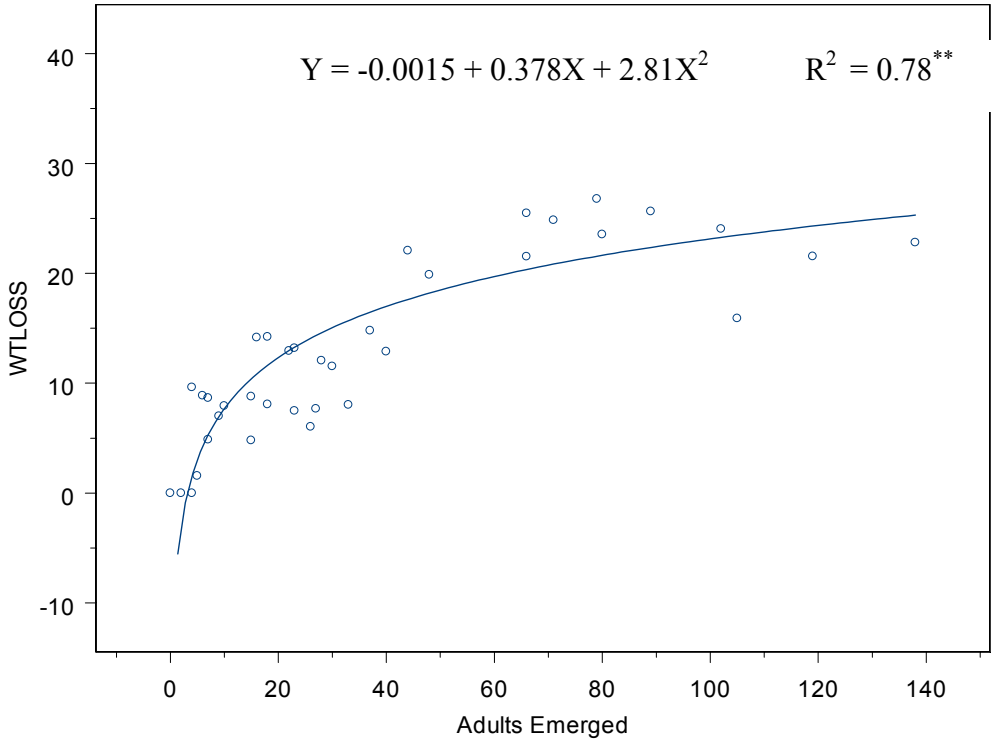

Figure 5.5. Relationship between percent seed weight loss (WTLOSS) and total number of F_1 emerging *A. obtectus* adults in introgression lines containing ARL proteins from tepary bean G40199.

General performance of ICA-Pijao and Rojo backcross lines to *A. obtectus* feeding

Seed damage was also reduced in backcross hybrids compared to the two susceptible parents as demonstrated by fewer seeds with 5 or more holes per seeds and reduced seed perforation (Fig. 5.6). Lowest mean number of seeds containing 5 or more holes, and percent mean seed weight loss was significantly lower in interspecific hybrid lines as compared to parental checks. While both parents provided plentiful resources for growth of larvae, percent perforation and severity of damage were greater in Rojo compared to ICA-Pijao, presumably because of the larger seed size of Rojo.

Rojo Rojo BC R26.5 ICA-Pijao BC 10.3,4 ICA Pijao

Figure 5.6. Levels of *A. obtectus* damage in resistant interspecific hybrid backcross lines and parents Rojo and ICA Pijao observed 72 days after inoculation. Parental Rojo on far left and ICA-Pijao on far right, interspecific counterparts on the inside left and right, respectively.

No adults of *A. obtectus* emerged from seed samples of the parent tepary bean G40199 after a period of 120 days. To determine if the testa in this accession was a factor in preventing the entry of hatched *A. obtectus* larvae into the seeds, the seed was decorticated then inoculated with adult bruchids. Signs of larvae penetration into the seeds were clearly visible as powdery/floury appearance on the seeds and no larvae were observed to remain outside the seeds (Fig. 5.7a). However, no adult emerged after 90

days of observation indicating that the seed coat was not a factor that prevented *A. obtectus* adult emergence. While most arcelin containing introgression lines showed some damage and adult insect emergence, a few of the introgression lines had no adult emergence. While the absence of emerged adults in interspecific lines may represent escapes, we did observe a floury appearance on the surface of the beans indicating larval penetration into the seeds had occurred. These interspecific introgression lines were kept for further screening and seed increase. Some introgression lines containing ARL proteins showed high number of late emerged adults that were observed as late as 90 days after inoculation (Fig. 5.7b and Appendix 5.8 & 5.9), but the emerged adults were remarkably small and appeared weak.

(a) (b)

Figure 5. 7. Larval damage to decorticated G40199 seeds and to intact seeds of backcross line ICA Pijao 10.3,4. Signs of larval drilling on seeds (a) of G40199 and (b) level of damage in ARL containing backcross line ICA 10.3,4 after 90 days of *A. obtectus* development during feeding trial.
Notice in (a), the small round pits on the surface of bean seeds due to drilling of larvae on the seeds, and (b) the small size of perforations from small emerging adults.

Effect of seeds containing ARL proteins to weight of F_1 adults

i) Reduced fresh weight of F_1 adults

In the daily process of counting of emerging F_1 adults, significant differences in size of emerging adults were observed (Fig.5.8). Earlier emerging F_1 adults from ICA Pijao, Rojo and introgression lines with null expression of ARL were consistently larger in size than the emerging adults from seeds with ARL. The mean fresh weight of F_1 adults emerging from arcelin containing seeds was significantly reduced about two-fold than adults emerging from non ARL protein seeds (Fig. 5.9).

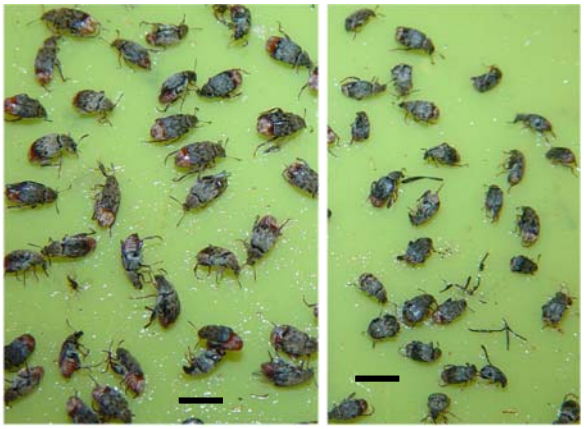

Figure 5.8. Size difference of F_1 *A. obtectus* adults emerging from interspecific hybrids seeds expressing ARL proteins (right) and from non ARL expressing seeds (left). A 5 mm bar is indicated by solid line.

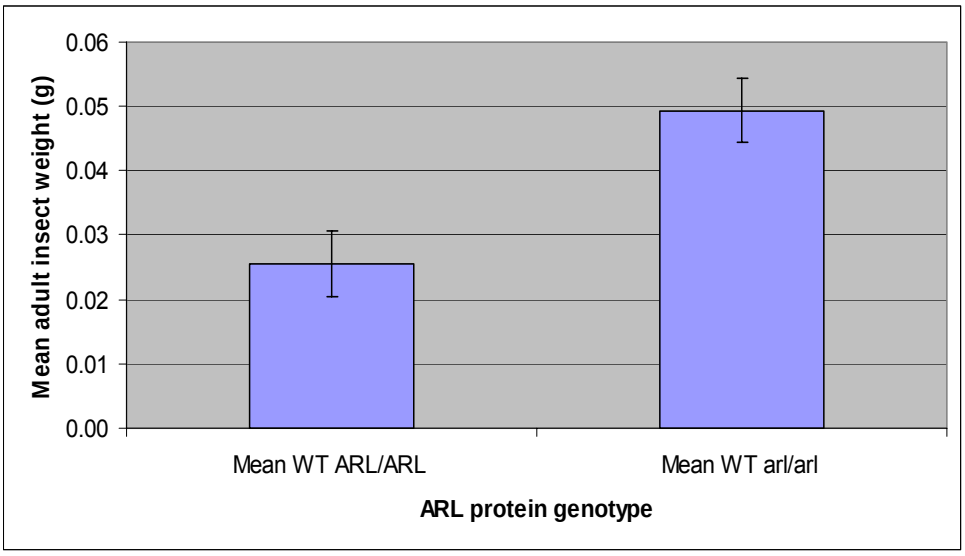

Figure 5. 9. Fresh weight (g) of F_1 *A. obtectus* adults collected from seeds with and without arcelin-ARL proteins of interspecific hybrids during feeding trials. Fifty four samples of 10 adults per sample were collected for both arcelin containing and arcelin null populations.

Effect of tepary ARL proteins into a phaseolin null background common bean

In an attempt to increase the proportion of arcelin proteins from tepary beans in introgression lines, we employed a strategy proposed by Hartweck et al. (1997) which involved introgression of ARL proteins into a phaseolin null background. Segregating lines for ARL proteins from accession G40199, *Arcelin-2* and phaseolin null were subjected to *A. obtectus* feeding trial. Significant resistance was observed among lines with phaseolin null but still segregating for *Arcelin-2* and ARL proteins as compared to seeds with phaseolins (Table 5.2). Although these findings were confounded by the segregating *Arcelin-2* background and prevented conclusions on the effectiveness of the ARL proteins alone in the absence of phaseolin, several lines of this cross combination demonstrated complete absence of damaged seeds by bruchids during the screening period (Appendix 5.8). Further selection for homozygous lines based on the expression of

ARL proteins is needed. Very few lines homozygous for ARL proteins and phaseolin null were obtained perhaps due to selection pressures against the arcelin-phaseolin null combination. These seeds will be increased and subjected to further screening against bruchids from tropical ecotypes.

Table 5.2. Levels of performance of introgression lines segregating for *arcelin* 2 (*Arl-2*^{pv}), ARL^{pa} proteins and phaseolin null from total seed storage proteins in a feeding trial against *A. obtectus*. N = 8 for *Phs*$^{-}$*Phs*$^{-}$, 20 for *Phs*$^{+}$*Phs*$^{-}$ and 15 for *Phs*$^{+}$*Phs*$^{+}$ classes.

	Bruchid F_1		Seeds			
Phaseolin genotype	50% emergence (Days)	Emerged (No.)	Perforated (%)	Severity >5 holes (No.)	SI^{z}	Weight loss (%)
Phs^{-} *Phs*$^{-}$	60^{A}	15^{B}	3.7^{B}	1.1^{B}	1.2^{C}	3.7^{C}
Phs^{+} *Phs*$^{-}$	59^{A}	30^{B}	7.3^{B}	2.8^{B}	1.9^{B}	7.4^{B}
Phs^{+} *Phs*$^{+}$	54^{B}	80^{A}	14.7^{A}	7.1^{A}	3.2^{A}	17.6^{A}
Overall means	58.6	34	7.4	3	1.9	8

Means with the same letter are not significantly different (Fisher's LSD of means) at α = 0.05. ^{z} Susceptibility index, *Phs*$^{-}$ *Phs*$^{-}$ = phaseolin null class, *Phs*$^{+}$ *Phs*$^{-}$ phaseolin segregating class, *Phs*$^{+}$ *Phs*$^{+}$ = class homozygous for phaseolin

DISCUSSION

Consistent resistance to *A. obtectus* was associated with the expression of the 33 kDa protein linked with the ARL proteins among introgression lines developed. This complex APA locus in G40199 includes ARL-3^{pa} and ARL-4^{pa}, phytohaemagglutinins and possibly α-AI-^{pa} as demonstrated in chapters 3 & 4. Homozygous lines for 33 kDa proteins had higher levels of resistance compared to the heterozygous genotypes in the two backcross populations. However, there were greater variations in emergence of adult bruchids among individual introgression lines homozygous for ARL proteins compared to susceptible lines (Figure 5.1; Appendix 5.9). If the resistance is strongly associated with the presence of the 33 kDa and the other linked subunits of lectin-like proteins (LLP) complex, then there may have been other factors that affected concentration, composition or conformation of the proteins. While some lines expressing the 33 kDa protein profiles were damaged with late emerging adults, some were completely not damaged during the feeding trial (Appendices 5.8 and 5.9), and even after being re-inoculated, only a few weak, late-emerging adults were observed. The delay in adult emergence among homozygous ARL containing introgression lines was remarkably high when compared to susceptible cultivars Rojo and ICA Pijao used as parents. A mean delay for 50% F_1 bruchid adult emergence of 63 days after inoculation among interspecific genotypes containing ARL proteins is a significant factor for bean storage in the tropical bean producing countries. In addition, the slow rate of adult emergence observed in ARL containing lines developed in this program is a significant factor for resistance to the bruchid *A. obtectus*. Some of the backcross interspecific lines had as much as a 69 days delay in emergence. Farmers in sub-Saharan Africa may store their

beans for 90 to 180 days between planting seasons (obviously, they may store for shorter periods of time if they sell the crop rather than keeping it for seed). For farmers in a bimodal rainfall climate who stores their beans for three to four months, there would be time for only one, or at most, two generations of bruchid reproduction. Thus, significant damage would only occur very late in storage, and farmers may be able to mitigate this with minimal integration of other pest management strategies for bruchids (Schoonhoven & Cardona, 1986; Mazzonetto and Vendramim, 2003) that will further prolong the developmental period and increase adult mortality. In addition to delayed emergence, emerging adults from these bean genotypes were fewer, small and most likely weak. While we did not quantify fitness of these beetles, we would expect that their reproductive fitness would be diminished.

The reduction of fresh F_1 adult weight in bruchids emerging from seeds containing homozygous ARL proteins is important supporting evidence that this protein is highly associated with resistance to *A. obtectus*. Poor digestibility of arcelin proteins by the insect's larval enzymes was observed in arcelin containing common bean (Minney et al. 1990; Goossens et al. 2000; Paes et al. 2000) that possibly contributed to adult size and weight reduction. The ARL proteins in introgression lines may have contributed to the disruption of the digestive system of the larvae thus affecting their development. The small-emerging adults may have suffered other physiological disabilities that contributed to the total delay in instar development, and reproductive disorders may be expected. Consequently, the subsequent insect generations may experience low fecundity and further weakening the entire bruchid colony in a given bean stock containing tepary ARL proteins.

Full resistance to *A. obtectus* was observed in wild tepary bean G40199 where no single adult emerged from either intact seeds or those with testa removed. The total absence of damaged seeds in the parent G40199 indicates that factors other than the 33 kDa of the ARL proteins may have increased antibiosis in this accession. When the total seed storage proteins from introgression lines and G40199 were compared by SDS-PAGE gels, the 26 kDa protein subunit was weak or missing in introgression lines, and 31 kDa band was modified into subunits (Chapter 4). These may represent modification of the composition or activity of protein subunits of the APA locus in the interspecific hybrids, resulting in different resistance levels. In addition, the amino acid sequence substitutions observed in the introgression lines as compared to the parent G40199 (Chapter 4) may be part of the reasons for the variable resistance observed. The introgression lines have high phaseolin expression levels which is the major seed storage protein donated from the recurrent parents. This protein may be an alternative nutrition source for the survival of feeding larvae in the seeds of introgression lines. Alternatively, the proportion of LLPs relative to other seed storage proteins in this accession may be significantly greater, and at levels that inhibit any development of larvae inside the seed. On SDS-PAGE gels, the 33 kDa protein band in G40199 was visually more intense than the phaseolin bands at ~ 45 kDa (Fig. 4.12 chapter 4), whereas introgression lines had a much more intense phaseolin band relative to the 33 kDa bands. Introgression lines with the 33 kDa proteins that demonstrated high levels of antibiosis to the bruchids may have a relatively higher expression of the protein to the level comparable to that of G40199.

Tepary beans possess other antinutritional proteins and non-protein compounds (Iduoraine et al. 1992; Magdi et al. 2003; Campos et al. 2004) that may play an additional

important role in antibiosis to bruchids. These proteins and other factors may not have been transferred into common bean by interspecific hybridization in the present situation. Lack of recombination, chromosome elimination, and loss of quantitative traits among interspecific hybrids in common bean x tepary bean crosses have been documented (Rabakoarihanta et al. 1979; Federici and Waines 1989; Mejia-Jiménez et al., 1994; Munõz et. al., 2004). As an approach to minimizing such problems, we are developing inbred congruity backcross lines of interspecific hybrids to G40199 containing two doses from *P. acutifolius* and *P. vulgaris* (ICA Pijao), respectively, followed by two selfing generations. These seeds were not used in this bruchid screening trial because few seeds are currently available.

Significant reduction in seed damage demonstrated by reduced seed weight loss and low number of severely perforated seeds with 5 or more holes was highly associated with the introgressed proteins among backcross families. Low damage was highly correlated with the reduced adult F_1 fresh weight, low susceptibility index, and reduced number of emerging F_1 adults.

Although the full resistance observed in G40199 was not transferred into two common bean lines by interspecific hybridization, the level of resistance reported in homozygous lines for the expression of ARL is highly significant when compared to the susceptible parent common bean cultivars Rojo and ICA Pijao. Delayed F_1 adult emergence, slow rate of emergence, reduced number of F_1 adults and reduction in size and weight of adults are important factors for bruchid resistance introgressed into common bean cultivars in this interspecific hybridization. Comparison of variables for levels of resistance observed in bruchid screening indicated that the two backcross lines were equally resistant and

superior to the parents regardless of the segregation of the ARL proteins. Bruchid screening confirmed the stability of the introgressed APA genes into common beans as suggested by the genomic analysis for expression of APA genes in backcross lines.

To our knowledge, this is the first work that describes the transfer and stable introgression into common bean cultivars and characterization of arcelin proteins from tepary bean. The highly resistant G40199 accession has been used by other researchers to study resistance to bruchid species and efforts to make interspecific hybrids with common beans have been attempted (Mejia-Jiménez et al. 2002; Cardona et al. 2005). However, no stable self reproductive plants have been developed with resistance to *A. obtectus*. Prior to this work, characterization of antibiosis factors of the lectin-like protein variants in this accession and their association to resistance to *A. obtectus* have not been documented. This is the second stable biotic resistance factor transferred from tepary bean to common bean cultivars in addition to resistance to common bean bacterial blight (*Xanthomonas campestris* pv. *phaseoli*) (Singh and Muñoz, 1999).

Germplasm of interspecific hybrids that demonstrate high levels of resistance to *A. obtectus* will be increased and shared to the bean improvement community for introgression of the resistance to common bean cultivars where bruchids are problematic. Congruity backcross lines that are fertile and express the LLPs from G40199 have been developed and will be increased for bruchid resistance screening. Prominently resistant CBC lines will be identified and used for bean improvement and breeding programs.

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CHAPTER 6

CONCLUSION

The two pronged approach of studying the heritability 33 kDa proteins from wild *P. acutifolius* accession G40199 crossed to a cultivated brown seeded tepary bean accession and the transfer of this protein into *P. vulgaris* cultivars was successful and provided significant genetic information that allowed designing a breeding strategy for populations of interspecific hybrids. This study demonstrated the successful transfer of the APA locus that harbors the lectin-like seed storage proteins from wild tepary beans into common bean cultivars.

Knowing that the candidate proteins of interest were inherited in a single gene Mendelian fashion, we introgressed the proteins into large seeded elite cultivars of common beans preferred in East Africa via interspecific hybrids as bridge parent. While understanding the problems associated with interspecific hybridization due to unusual chromosome recombination and infertility of hybrids we developed congruity backcross (CBC) lines in order to enhance the number of loci that can be moved from *P. acutifolius* into *P. vulgaris*. These CBC lines were developed as a back-up in case the regular backcrossing procedures caused severe attenuation of the trait of interest. These lines will also be of importance in bean improvement for other traits of tepary bean such as drought tolerance.

Genomic characterization of the composition of the lectin-like proteins in the accession G40199, and the Brown Tepary bean accession demonstrated the apparent presence of the APA locus in the two *P. acutifolius* accessions. This characterization allowed us to identify similar lectin-like proteins (ARL, PHA and α-AIs) in interspecific hybrids generated from the cross, thus confirming the reliability of interspecific hybrids with introgression of the lectin-related genes. Successful introgression of a 33 kDa and

other protein profiles linked with arcelin variants has been demonstrated in the interspecific hybrids by expression and proteomic studies of the expressed proteins both in young immature and dry seeds respectively.

Several peptides were observed in the parent G40199 and the derived interspecific hybrids demonstrating the presence of two arcelin variants and a phytohaemagglutinin in a single genotype as important proteins for antibiosis. A combination of proteomic MS-MS and mRNA characterization allowed us to identify the composition of lectin-like proteins residing in tepary beans and determine their evolutionary relationships with other lectin related proteins in common beans. New variants of genes for arcelins (ARL-3^{pa} and ARL-4^{pa}) have been observed in the accession G40199 and were successfully introgressed into cultivars of common bean by interspecific hybridization via normal backcrossing procedures. The novel partial gene sequences isolated and characterized from G40199 will be a great contribution to the bean improvement community and insect research in general. The sequences will be deposited into a gene database for public use. However, full gene sequences for these genes need to be developed as cloned sequences. Meanwhile, DNA markers specific to these genes and the 33 kDa will be useful for selection of resistant lines in a bruchid resistance breeding program to incorporate these genes into other common bean cultivars.

The stable transfer of these genes into common bean cultivars has been demonstrated among several inbred backcross lines into three main *P. vulgaris* backgrounds; the bridge parent ICA-Pijao, Rojo and a Rojo backcross with *Arcelin 2*+ phaseolin null backgrounds. The evaluation of the association of the introgressed ARL proteins from tepary beans to *A. obtectus* resistance among the interspecific backcross

hybrid lines has demonstrated resistance to the bruchid by delayed days for adult emergence, reduced rate of adult emergence and reduced size/weight of adults in the presence of the proteins. This is an important factor for resistance as it contributes to reduced fitness of the bruchid colony infecting the same seed lot and consequently lowers seed damage. The accession G40199 was completely resistant to the bruchid as no emerging F_1 adults were observed in several bruchid feeding trials conducted in this work at OSU and in Malawi. Researchers at CIAT, Goossens et al. (2000) reported adult emergence from this accession, which raises questions whether the accession is variable or the difference is due to different ecotypes of the bruchid species.

This work produced common bean lines that have significant resistance to *A. obtectus* reported to be associated with arcelins from *P. acutifolius*. Stable and consistent resistance was associated with the presence of the introgressed LLPs into some of the lines with two generations of backcross to the adapted elite cultivar Rojo and three selfing crosses. These lines will provide an important contribution to bruchid resistance in East Africa and will need one more backcrossing and evaluation for resistance and other agronomic performance to be developed into a near-isogenic line. Introgression of the APA locus into phaseolin null background of common bean cultivars remains as another important strategy to enhance resistance to bruchids as demonstrated in some of the phaseolin-null materials in this study. Prominent bruchid resistant lines observed in this study using the bruchid colony here will be increased and used for further bruchid testing on the tropical ecotypes of *A. obtectus* and *Z. subfasciatus* in Africa and other places where bruchids are a problem in bean storage. The lines developed here will serve as germplasm with *P. acutifolius* introgression useful for genetic improvement and be

used as future bridge parents for interspecific hybridization with other accessions of *P. acutifolius* to cultivars of *P. vulgaris*.

The successful results of interspecific hybridization and transfer of resistance to bruchids in this work demonstrate the need for continuous use of other accessions of *P. acutifolius* for interspecific hybridization to incorporate other important agronomic traits residing in tepary beans. These kinds of works are not now commonly carried out by researchers in bean improvement in the fear of the challenges of incompatibility, and as a result, only a few traits have been moved from *P. acutifolius* into *P. vulgaris*. This work describes the second successful transfer of genetic resistance from tepary bean into common bean in addition to common bean bacterial blight resistance. These results are encouraging and could stimulate more work to identify new arcelin variants in tepary bean, interspecific hybridization and further studies of the introgressed novel arcelin variants in order to understand the genetic structure of the APA locus in accession G40199.

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APPENDICES

Appendix 3.1 Pod and seed set characteristics of interspecific backcross lines generated from the cross ICA Pijao x G40199.

	Pod and Seed set characteristics				
Cross ID	**Empty pods**	**1 seed**	**2- 3 seeds**	**> 3 seeds**	**Maximum seeds/pod**
			No.		
CBC1F2 ICA X G40199-1	0	3	21	4	4
CBC1F2 ICA X G40199-2	80	21	2	0	2
BC1F2 ICA 6.2	19	68	25	0	3
BC1F2 ICA 7.3	4	25	10	0	2
BC1F2 ICA 7.3.1	3	20	2	0	2
BC1F2 ICA 12.1	6	42	33	4	4
BC1F2 ICA 12.2	5	17	0	0	1
BC1F2 ICA 16.1	0	1	8	38	7
BC1F2 ICA 19.3	63	17	0	0	1
BC1F2 ICA 28.1	18	33	0	0	1
BC1F2 ICA 28.2/05	7	65	62	5	4
BC1F2 ICA 37.1	28	27	3	0	2
BC1F2 ICA 38.1	7	22	1	0	2
BC2F2 ICA 43.3	4	0	3	35	8
BC2F2 ICA 43.4	0	1	2	33	8
BC2F2 ICA 43.5	0	0	3	41	8
BC2F2 ICA 43.6	0	0	2	36	8
BC2F2 ICA 43.9	0	2	6	22	8
BC2F2 BTICA 1.2	50	47	6	0	2
BC2F2 BTICA 1.3	0	0	6	28	8
BC2F2 BTICA 1.4	2	21	52	21	6
BC2F2 BTICA 1.5	6	33	34	2	4
BC2F2 BTICA 1.6	4	18	26	42	6

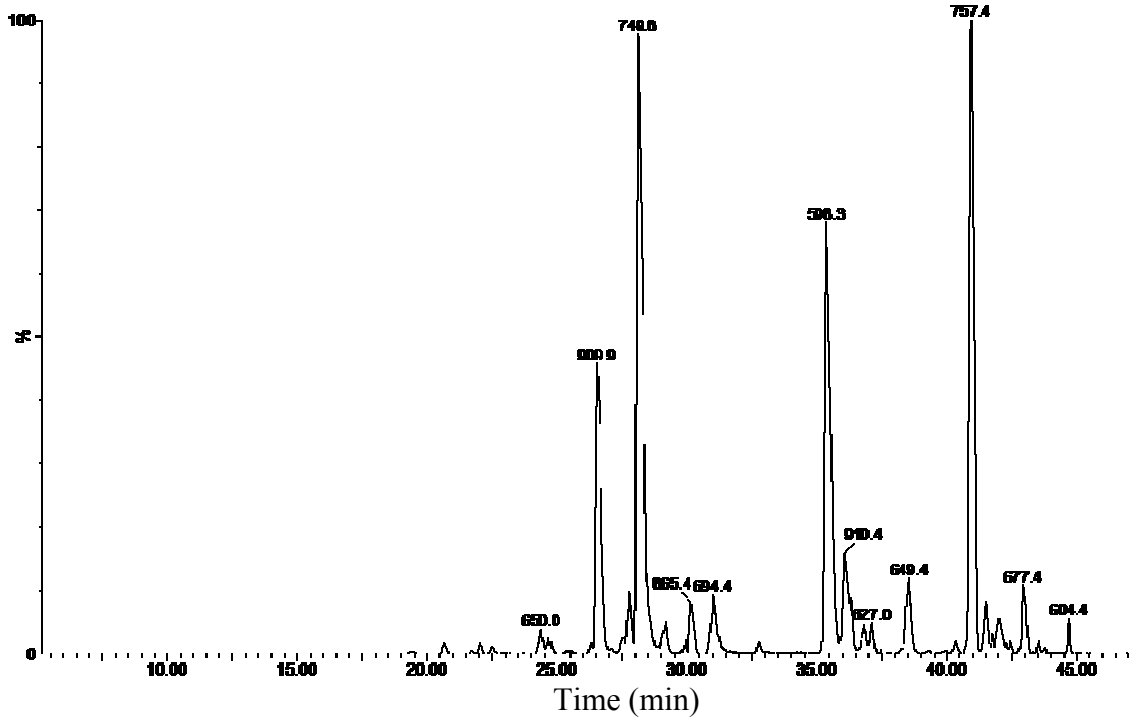
Appendix 3.1 (continued)

	Pod and Seed set characteristics				
Cross ID	**Empty pods**	**1 seed**	**2- 3 seeds**	**> 3 seeds**	**Maximum seeds/pod**
			No.		
BC2F2 BTICA 1.7	0	3	19	24	8
BC2F2 BTICA 1.8	32	22	3	0	2
BC2F2 BTICA 1.9	0	10	23	3	5
BC2F2 BTICA 1.10	5	34	7	0	3
BC2F2 BTICA 1.11	29	31	8	0	3
BC2F2 BTICA 1.2.1	3	11	30	9	6
BC2F2 BTICA 1.2.4	41	26	3	0	2
BC2F2 BTICA 1.2.5	0	2	0	32	8
BC2F2 BTICA 1.2.6	3	43	21	2	6
BC2F2 BTICA 1.2.7	2	3	23	10	5
BC2F2 BTICA 1.2.8	12	36	19	1	4
BC2F2 BTICA 1.2.9	16	50	25	2	4
BC2F2 ICA 7.2.1	0	0	2	28	7
BC2F2 ICA 7.2.2	0	0	3	50	7
BC2F2 ICA 7.2.4	0	0	6	38	7
BC2F2 ICA 7.2.6	1	10	16	12	6
BC2F2 ICA 7.2.10	0	1	10	28	8
BC2F2 ICA 7.2.12	0	1	9	41	8
BC2F2 ICA 7.2.16	0	0	3	41	8

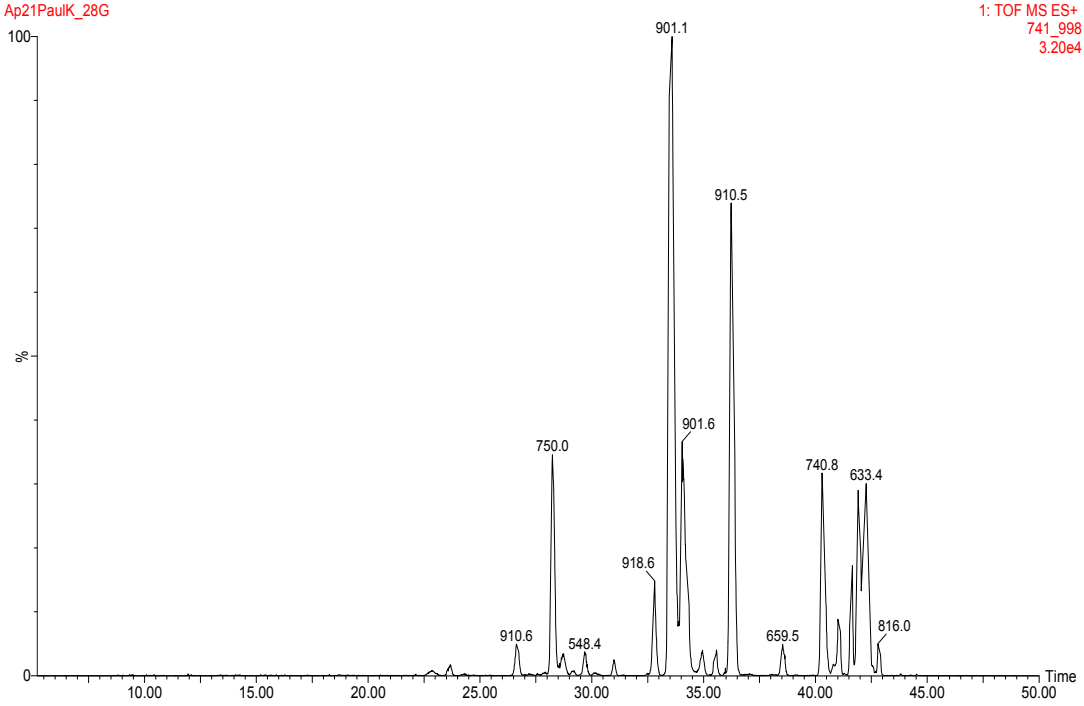
Appendix 3.2. Genomic DNA sequence alignment of α-AI[pa] sequence from Brown Tepary bean to α-AI[pa] *P. acutifolius*

```
α-AI-pa          1  ATGGCTTCCTCCAAGTTCTGCAGTGTGCTCTCCCTAGTCCTCTTCCTTGT-----GCTTC 60
α-AIpa-BT             ----GTTCCTCCAAGT-CTGCAGTGT-CTCTGCCTAGTCCTCTTCCTTGTCTTGTGCTTC
                           *********** ********* **** ******************     *****
α-AI-pa                TCACCCACGCAAACTCAGCCTGCAACACCTCCTTCAACTTCCATAGTTTCAACGAAACCA 120
α-AIpa-BT              TCACCCACGCAAACTCAGCCAGCGACACC---TTCAACTTCCATAGTTTCAACGAAACCA
                       ******************* ** *****   ****************************
α-AI-pa                ACCTTATGCTTCAAGGCCAAGCCACCGTCTCATCCAACGGCAACTTACAACTAAATACAA 180
α-AIpa-BT              ACCTTATCCTCCAAGGCGATGCCACCGTCTCATCCAACGGCAACTTACAACTACATACAA
                       ******* ** ****** * ********************************* ******
α-AI-pa                TGGACTCTATGTGCAGCGCCTTCTACTCCGCCCCCATCCAAATCAGGGACAGCACCACCG 240
α-AIpa-BT              TGGACTCTATGTGCAGCGCCTTCTACTCCGCCCCCATCCAAATCAGGGACAGCACCACCG
                       ************************************************************
α-AI-pa                GCAACGTTGCCAGCTTCGACACCAACTTCACAATCAATATGACCAGTTATTGCAAAGCAA 300
α-AIpa-BT              GCAACGTCGCCAGCTTCCACACCAACTTCACAATGAATATCACCACTTACCGCAAAGCAA
                       ******* ********* *************** ***** **** ***  *********
α-AI-pa                ATTCCGCCGTTGGCCTTGACTTTGCTCTCGTCCCCGTCCAGCCCAAATCCAAAGGCCGTC 360
α-AIpa-BT              ATTCCGCCGTTGGCCTAGACTTTGCTCTCGTCCCCGTCCAGCCCAAATCCAAAGGCCGTC
                       *************** ******************************************
α-AI-pa                TTCTAGGTCTTTTCAAGACACCCGACTACGACAGAAACGCCGGTAATGTGACTGTGGAGT 420
α-AIpa-BT              TTCTAGGTCTTTTCAAGACACCCGACTACGACAGAAACGCCGGTATTGTGACTGTGGAGT
                       ****************************************** **************
α-AI-pa                TCGACACCTTCCGCAGACGTATTAGCATCGACGGGAACCATAACGATATCGAAAGCGTGC 480
α-AIpa-BT              TCGACACCTTACGCAGACGTATTAGCATCGACGGGAACTACAATGATATCGAAAGCGTGC
                       ********** *************************** * ** ****************
α-AI-pa                CTTGGGATGTAGACGACTACGACGGACAAAACGCCGAGGTTCGGATCACCTATAACTCCT 540
α-AIpa-BT              CTTGGAATGTAGACGACTACGACGGACAAAAAGCCGAGGTTCGGATCACCTATAACTCCT
                       ***** ************************ ****************************
α-AI-pa                CCACGAAGGTCTTGGCGGTTTCTCTGTTAAACCTTTCTACGGGAAAGAGCAACAACGTCT 600
α-AIpa-BT              CCACGAAGGTCTTGGCGGTTTCTCTGTTAAACCCTTCTACGGGAAAGAGCAACAACGTCT
                       ******************************** ************************
α-AI-pa                CTGCCAGAATGGAGCTGGAGAAAAAACTTGACGACTGGGTGAGCGTTGGGTTCATTGGCA 660
α-AIpa-BT              CTGCCAGAATGGAGCTGGAGAAAAAACTTGACGACTGGGTGAGCGTTGGGTTCATTGGCA
                       ************************************************************
α-AI-pa                CCTCAGGGGTTCATCAATATAGCTTTGAAA-CGAGAGACGTGTTCTCTTGGTCTTTTTCT 720
α-AIpa-BT              CCTCAGGGGTTCATGAATATAGCTTTGAAAACGAGAGACGTGTTCTCTTGGTCTTTTTCT
                       ************** *************** ****************************
α-AI-pa                TCTAAGTTCTCCCAACACACCACATCTGAACGTTCCAACATCCTCATCAACCAAATCCTC 780
α-AIpa-BT              TCTAAGTTCTCCCAACACACCACATCT-----TTCCAACA--------------------
                       *************************     ********
α–AI–pa                TAG 790
a-AIpa-BT              ---
```

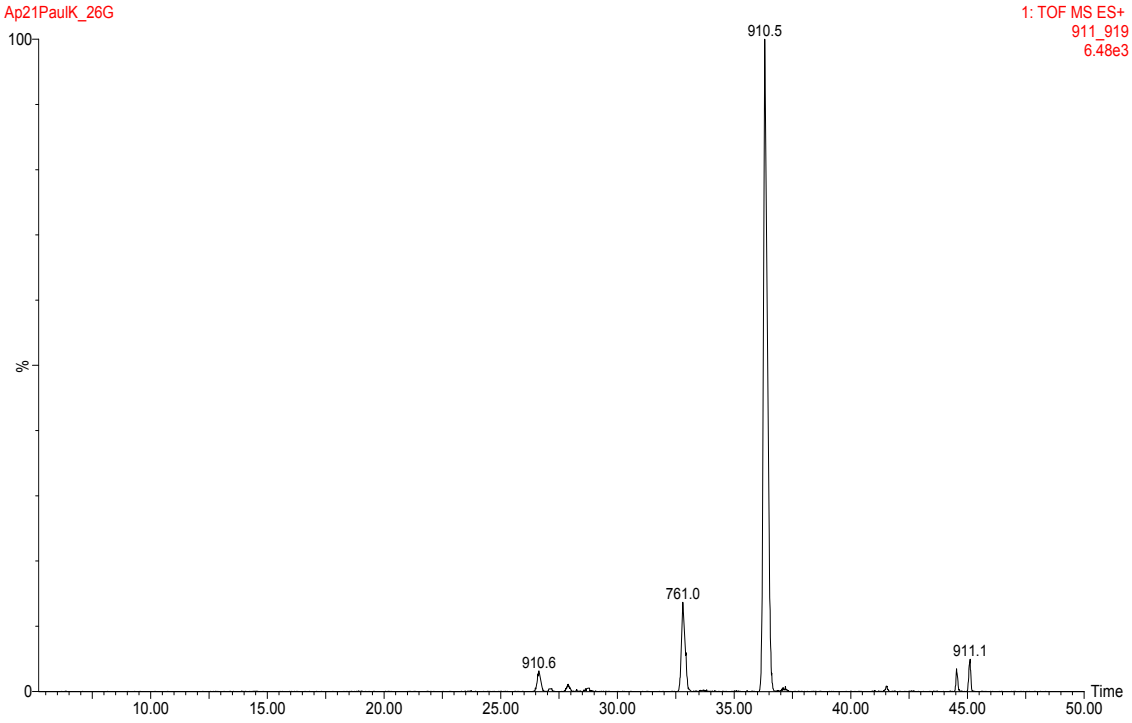
Appendix 4. 1. Chromatogram of m/z for protein peptide peaks produced by Q-TOF-ESI of the 31kDa protein fragment from total seed proteins of *P. acutifolius* accession G40199.


100
%
0
757.4
749.6
536.3
699.9
650.0
665.4
694.4
710.4
649.4
677.4
627.0
604.4
10.00
15.00
20.00
25.00
30.00
35.00
40.00
45.00
Time (min)

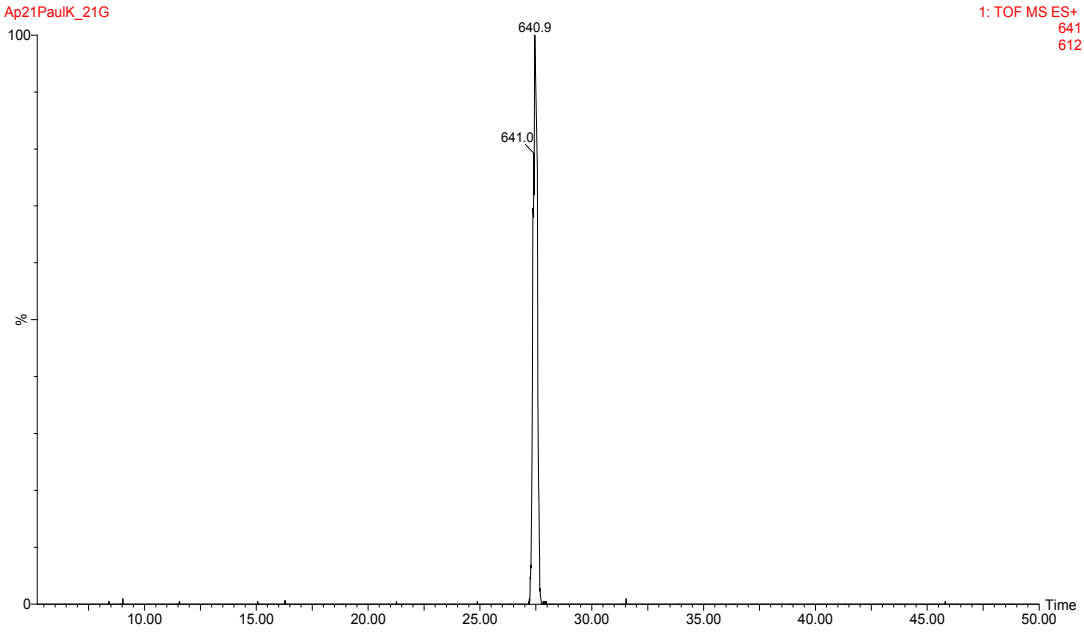
Appendix 4.2. Chromatogram of m/z for protein parent ions of peptide peaks produced by Q-TOF- ESI of the 28kDa protein fragment from total seed proteins of *P. acutifolius* accession G40199.



Appendix 4.3. Chromatogram of m/z for protein peptide peaks produced by Q-TOF- ESI of the 26kDa protein fragment from total seed proteins of *P. acutifolius* accession G40199



Appendix 4.4. Chromatogram of m/z for protein peptide peaks produced by Q-TOF- ESI of the 21kDa protein fragment from total seed proteins of wild tepary bean accession G40199



Appendix 5. 1 Analysis of variance for 50% F_1 adult emergence for two introgression families (BC Pop) and three genotypes (ARL/ARL, ARL/arl, arl/arl) in *A. obtectus* feeding trials. Number of eggs was used as a covariate.

Sources of Variation	Degree of Freedom	Sum of Squares	Mean Square	F Value	Pr > F
BC Pop	1	10.456	10.456	0.58	0.4465
Genotypes	2	3676.749	1838.375	102.56	<.0001
No. Eggs	1	0.049	0.049	0.00	0.9585

R-Square	CV	Root MSE	DAE50% Mean
0.63	7.97	4.23	53.11

Appendix 5. 2. Analysis of variance for total bruchid F_1 adult emergence for two introgression families (BC Pop) and three genotypes (ARL/ARL, ARL/arl, arl/arl) in *A. obtectus* feeding trials. Number of eggs was used as a covariate.

Sources of Variation	Degree of Freedom	Sum of Squares	Mean Square	F Value	Pr > F
BC Pop	1	41297.591	41297.591	93.62	<.0001
Genotypes	2	30500.248	15250.124	34.57	<.0001
No. Eggs	1	76695.676	76695.676	173.86	<.0001

R-Square	CV	Root MSE	Adult emerged Mean
0.66	53.78	21.00	39.05

Appendix 5. 3. Analysis of variance for percentage of perforated seeds for two introgression families (BC Pop) and three genotypes (ARL/ARL, ARL/arl, arl/arl) in *A. obtectus* feeding trials. Number of eggs was used as a covariate.

Sources of Variation	Degree of Freedom	Sum of Squares	Mean Square	F Value	Pr > F
BC Pop	1	415.301	415.301	1.78	0.1849
Genotypes	2	6399.653	3199.826	13.70	<.0001
No. Eggs	1	15363.035	15363.034	65.76	<.0001

R-Square	CV	Root MSE	Percent perforated Mean
0.44	46.1	15.28	33.14

Appendix 5. 4. Analysis of variance for severity of seed damage (> 5 holes/seed) for two introgression families (BC Pop) and three genotypes (ARL/ARL, ARL/arl, arl/arl) in *A. obtectus* feeding trials. Number of eggs was used as a covariate.

Sources of Variation	Degree of Freedom	Sum of Squares	Mean Square	F Value	Pr > F
BC Pop	1	255.1486345	255.1486345	26.50	<.0001
Genotypes	2	411.6537676	205.8268838	21.38	<.0001
# of Eggs	1	614.8222901	614.8222901	63.85	<.0001

R-Square	CV	Root MSE	Severity Mean
0.46	90.7	3.10	3.4

Appendix 5. 5 Analysis of variance for susceptibility index (SI) for two introgression families (BC Pop) and three genotypes (ARL/ARL, ARL/arl, arl/arl) in *A. obtectus* feeding trials. Number of eggs was used as a covariate.

Sources of Variation	Degree of Freedom	Sum of Squares	Mean Square	F Value	Pr > F
BC Pop	1	19.290	19.290	55.11	<.0001
Genotypes	2	61.318	30.659	87.60	<.0001
No. Eggs	1	28.937	28.937	82.68	<.0001

R-Square	CV	Root MSE	SI Mean
0.68	21.7	0.59	2.7

Appendix 5. 6. Analysis of variance for percent seed weight loss for two introgression families (BC Pop) and three genotypes (ARL/ARL, ARL/arl, arl/arl) in *A. obtectus* feeding trials. Number of eggs was used as a covariate.

Sources of Variation	Degree of Freedom	Sum of Squares	Mean Square	F Value	Pr > F
BC Pop	1	418.197	418.197	10.77	0.0014
Genotypes	2	1997.917	998.958	25.71	<.0001
No. Eggs	1	2128.333	2128.333	54.79	<.0001

R-Square	CV	Root MSE	Percent WT loss Mean
0.47	57.9	6.23	10.767

Appendix 5. 7. Analysis of variance of fresh weight of F_1 *A. obtectus* adults collected from seeds with and without ARL proteins of introgression lines during bruchid feeding trials.

Sources of Variation	Degree of Freedom	Sum of Squares	Mean Square	F Value	Pr > F
ARLTYPE	1	0.0142	0.0142	645.02	<.0001

R-Square	CV	Root MSE	Adult WT Mean
0.86	12.8	0.0047	0.036635

Appendix 5. 8. Levels of performance of introgression lines segregating for arcelin 2 (*Arl-2*^{pv}) , ARL proteins and phaseolin null (*Phs*) from total seeds storage proteins against *A. obtectus* observed in 90 days from adult infestation.

Phaseolin genotype	Vial No.	Arcelin allele present	WT loss (%)	Perforated seeds (%)	Severity[z]	Adult Emergence (no.)	SI[y]	DAE 50%	EGGS (no.)
Phs-Phs-	16-2	ARL^{pa}	2.31	6.7	1	6	1.3	59	68
Phs-Phs-	16-4	ARL^{pa}	0.00	0.0	0	0	0.0		58
Phs-Phs-	16-6	ARL^{pa}	6.87	26.7	0	18	2.1	59	76
Phs-Phs-	13-8	ARL^{pa}	0.38	0.0	0	0			
Phs-Phs-	13-5	ARL^{pa}	0.82	3.3	0	1	0.0	63	78
Phs-Phs-	13-3	ARL^{pa}	1.81	10.0	0	3	0.8	61	60
Phs-Phs-	16-1	ARL^{pa}	2.52	20.0	0	11	1.7	62	75
Phs-Phs-	16-4	ARL^{pa}	5.30	16.7	2	21	2.2	59	76
Phs-Phs-	16-5	ARL^{pa}	4.39	13.3	0	5	1.2	59	79
Phs-Phs-	16-6	ARL^{pa}	5.84	16.7	1	22	2.2	60	98
Phs-/Phs+	18-4	ARL^{pa}	0.00	0.0	0	0			78
Phs-/Phs+	13-2	ARL^{pa}	3.25	10.0	1	14	2.1	55	74
Phs-/Phs+	13-4	ARL^{pa}	0.12	0.0	0	0			48
Phs-/Phs+	16-3	ARL^{pa}	10.57	23.3	3	38	2.4	65	60
Phs-/Phs+	18-1	ARL^{pa}	10.93	53.3	3	40	2.7	59.5	60
Phs-/Phs+	18-2	ARL^{pa}	7.01	23.3	1	21	2.2	60.5	94
Phs-/Phs+	18-6	ARL^{pa}	10.24	30.0	0	25	2.1	65.5	62
Phs-/Phs+	13-1	ARL^{pa}	23.39	70.0	10	68	3.3	56	89
Phs-Phs-	14-5	Alr-2 ^{pv}	0.00	0.0	0	0			56
Phs-Phs-	12-11	Alr-2 ^{pv}	20.48	50.0	10	101	3.5	57.5	78
Phs-Phs-	16-7	Alr-2 ^{pv}	0.00	0.0	0	0	0.0	70	43
Phs-Phs-	14-2	Alr-2 ^{pv}	0.63	0.0	0	0			42
Phs-Phs-	14-6	Alr-2 ^{pv}	0.52	3.3	0	1	0.0	58	45
Phs-/Phs+	14-4	Alr-2 ^{pv}	0.00	0.0	0	0			65
Phs-/Phs+	14-8	Alr-2 ^{pv}	1.56	3.3	0	3	0.8	59	73

[z] Number of seeds with greater than five perforations. [y] Susceptibility Index

Appendix 5.8 continued

Phaseolin genotype	Vial No.	Arcelin allele present	WT loss (%)	Perforated seeds (%)	Severity[z]	Adult Emergence (no.)	SI[y]	DAE 50%	EGGS (no.)
Phs-/Phs+	14-1	Alr-2^{pv}	0.71	0.0	0	0	0.0	70	58
Phs-/Phs+	14-7	Alr-2 ^{pv}	0.53	0.0	0	0	0.0		70
Phs-/Phs+	12-6	Alr-2 ^{pv}	29.42	60.0	14	121	4.3	48	116
Phs-/Phs+	12-7	Alr-2 ^{pv}	6.44	36.7	3	27	2.4	59	56
Phs-/Phs+	12-9	Alr-2 ^{pv}	21.53	70.0	15	135	4.0	53	108
Phs-/Phs+	12-1	Alr-2 ^{pv}	3.28	13.3	0	10	1.7	59	56
Phs-/Phs+	18-3	Alr-2 ^{pv}	5.23	30.0	1	21	2.2	59	94
Phs-/Phs+	18.5.1	Alr-2 ^{pv}	7.35	26.7	1	26	2.4	58.5	56
Phs-/Phs+	18.5.2	Alr-2 ^{pv}	15.20	46.7	6	59	2.9	59.5	77
Phs-/Phs+	18-7	Alr-2 ^{pv}	10.40	36.7	5	45	2.6	63.5	24
Phs-/Phs+	18-8	Alr-2 ^{pv}	4.61	23.3	1	20	2.2	59.5	67
Phs+Phs+	14-9	Alr-2 ^{pv}	2.46	13.3	0	7	1.5	57	28
Phs+Phs+	14-3	Alr-2 ^{pv}	3.10	13.3	1	15	2.1	57	24
Phs+Phs+	12-8	Alr-2 ^{pv}	18.10	56.7	7	91	3.2	62	90
Phs+Phs+	12-4	Alr-2 ^{pv}	22.55	56.7	8	101	3.4	58.4	122
Phs+Phs+	12-5	Alr-2 ^{pv}	28.28	66.7	14	138	4.7	45.5	132
Phs+Phs+	12-1	Alr-2 ^{pv}	15.22	40.0	5	54	3.4	51	96
Phs+Phs+	12-2	Alr-2 ^{pv}	27.79	76.7	12	130	4.4	48.5	102

[z] Number of seeds with greater than five perforations. [y] Susceptibility Index.

Appendix 5. 9 Levels of performance of Rojo and ICA Pijao backcross lines from introgression lines segregating for *P. acutifolius* ARL seed storage proteins in a feeding trial with *A. obtectus* observed after 90 days of adult infestation.

Backcross	Line/Vial No.	Arcelin allele present	Adult Emergence (no.)	SI[y]	Severity[z]	WT loss (%)	DAE 50%	Perforated seeds (%.)	EGGS (no.)
RojoBC2F2:3	R26-1	ARL/ARL	4	1.08	0	0.00	56.0	0.0	112
RojoBC2F2:3	R48-4	ARL/ARL	0	0.00	0	0.00		0.0	104
RojoBC2F2:3	R26-5	ARL/ARL	4	0.89	0	9.64	68.0	30.0	150
RojoBC2F2:3	R38-13	ARL/ARL	37	2.70	3	14.79	58.0	55.0	162
RojoBC2F2:3	R48-20	ARL/ARL	10	1.67	0	7.94	60.0	25.0	92
RojoBC2F2:3	R48-7	ARL/ARL	9	1.54	0	7.01	62.0	20.0	110
RojoBC2F2:3	R48-8	ARL/ARL	7	1.32	1	4.86	64.0	10.0	100
RojoBC2F2:3	R48-23	ARL/ARL	7	1.30	1	8.68	65.0	25.0	115
RojoBC2F2:3	R48-25	ARL/ARL	66	3.08	11	25.47	59.0	68.4	120
RojoBC2F2:3	R38-17-1	ARL/ARL	44	2.57	5	22.08	64.0	55.0	154
RojoBC2F2:3	R38-17-2	ARL/ARL	6	1.13	2	8.87	69.0	20.0	135
RojoBC2F2:3	R26-8	ARL/arl	2	0.63	0	0.00	48.0	0.0	73
RojoBC2F2:3	R26-2	ARL/arl	27	2.68	5	7.68	53.5	25.0	103
RojoBC2F2:3	R26-9	ARL/arl	33	2.92	2	8.04	52.0	45.0	136
RojoBC2F2:3	R26-11	ARL/arl	26	2.86	1	6.04	49.5	35.0	68
RojoBC2F2:3	R26-12	ARL/arl	28	2.76	1	12.06	52.5	30.0	134
RojoBC2F2:3	R38-15	ARL/arl	71	3.28	8	24.84	56.5	70.0	130
RojoBC2F2:3	R48-3	ARL/arl	40	3.08	4	12.88	52.0	40.0	92
RojoBC2F2:3	R48-9	ARL/arl	5	1.43	0	1.57	49.0	10.0	82
RojoBC2F2:3	R48-14	ARL/arl	80	3.66	8	23.55	52.0	75.0	98
RojoBC2F2:3	R48-15	ARL/arl	15	2.26	0	4.81	52.0	20.0	68
RojoBC2F2:3	R38-5	arl/arl	18	2.64	0	14.23	47.5	40.0	52
RojoBC2F2:3	R38-10-1	arl/arl	23	2.81	3	7.50	48.5	20.0	70
RojoBC2F2:3	R38-10-2	arl/arl	23	2.93	3	13.19	46.5	30.0	65

[z] Number of seeds with greater than five perforations. [y] Susceptibility Index.

Appendix 5.9 continued

Backcross	Line/Vial No.	Arcelin allele present	Adult Emergence (no.)	SI[y]	Severity[z]	WT loss (%)	DAE 50%	Perforated seeds (%.)	EGGS (no.)
RojoBC2F2:3	R38-22	arl/arl	16	2.41	0	14.16	50.0	20.0	74
RojoBC2F2:3	R38-16	arl/arl	48	3.26	5	19.88	51.5	50.0	153
RojoBC2F2:3	R48-1	arl/arl	22	2.98	2	12.94	45.0	25.0	88
RojoBC2F2:3	R48-2	arl/arl	30	2.87	5	11.54	51.5	30.0	80
RojoBC2F2:3	R48-11	arl/arl	15	2.42	0	8.80	48.5	35.0	109
RojoBC2F2:3	R48-12	arl/arl	79	3.61	7	26.79	52.5	40.0	116
RojoBC2F2:3	R48-13	arl/arl	66	4.00	8	21.54	45.5	55.0	106
RojoBC2F2:3	R48-17	arl/arl	18	2.73	3	8.08	46.0	25.0	78
RojoBC2F2:3	R38-1.1	arl/arl	89	4.48	13	25.65	43.5	90.0	112
RojoBC2F2:3	R38-1.2	arl/arl	134	4.25	15	39.19	50.0	75.0	154
Rojo	Rojo-1	arl/arl	138	5.04	11	22.82	42.5	65.0	148
Rojo	Rojo-2	arl/arl	105	4.87	8	15.91	41.5	86.4	110
Rojo	Rojo-3	arl/arl	119	4.51	12	21.55	46.0	80.0	130
Rojo	Rojo-4	arl/arl	102	4.18	11	24.05	48.0	80.0	104
184/5-593	184/5-593	ARL/ARL	21	2.24	0	2.92	59	22.5	31
228/ICA 43.4	228.5	ARL/ARL	8	1.35	1	1.78	67	10.0	44
228/ICA 43.4	228.6	ARL/ARL	4	0.94	0	0.11	64	5.0	19
228/ICA 43.4	228.7	ARL/ARL	12	1.80	0	1.74	60	15.0	25
16/ICA7.2.10	16.5	ARL/ARL	2	0.44	0	6.16	68	27.5	17
222/ICA38.1	222.4	ARL/ARL	10	1.72	0	6.21	58	15.0	32
132/BT1.2.4	132.3	ARL/ARL	18	2.09	0	2.68	60	16.7	17
132/BT1.2.4	132.4	ARL/ARL	2	0.40	0	2.21	75	2.5	76
12/ICA 7.2.12	12.7	ARL/ARL	2	0.52	0	1.62	58	5.0	16
10/ICA43.9	10.1, .2II	ARL/ARL	45	2.76	2	18.99	60	45.0	68
10/ICA43.9	10.1,.2 I	ARL/ARL	58	2.94	7	24.88	60	60.0	70
10/ICA43.9	10.3,10.4	ARL/ARL	8	1.39	0	3.33	65	7.5	16

[z] Number of seeds with greater than five perforations. [y] Susceptibility Index.

Appendix 5.9 continued

Backcross	Line/Vial No.	Arcelin allele present	Adult Emergence (no.)	SI[y]	Severity[z]	WT loss (%)	DAE 50%	Perforated seeds (%.)	EGGS (no.)
10/ICA43.9	10.5	ARL/ARL	40	2.50	5	11.22	64	52.5	70
10/ICA43.9	10.6, .7	ARL/ARL	30	2.20	3	9.34	67	45.0	77
4/ICA 43.4	4.7	ARL/ARL	11	1.68	0	1.28	62	15.0	42
4/ICA 43.4	4.2	ARL/arl	123	3.77	13	20.71	55.5	67.5	160
4/ICA 43.4	4.3	ARL/arl	28	2.58	3	6.03	56	22.5	95
4/ICA 43.4	4.4	ARL/arl	70	3.21	6	16.02	57.5	52.5	122
4/ICA 43.4	4.6	ARL/arl	50	2.79	5	10.04	61	52.5	85
5/ICA 7.2.6	5.1 5.3	ARL/arl	9	1.60	0	16.21	59.5	17.5	19
5/ICA 7.2.6	5.4 5.5	ARL/arl	91	3.50	8	21.56	56	50.0	89
5/ICA 7.2.6	5.5 5.7	ARL/arl	38	2.72	2	9.81	58	42.5	47
6/ICA7.2.16	6.4	ARL/arl	8	1.81	0	4.92	50	0.0	30
6/ICA7.2.16	6.6	ARL/arl	15	2.67	4	8.85	44	20.0	48
6/ICA7.2.16	6.7	ARL/arl	40	3.11	3	7.66	51.5	27.5	63
8/ICA 43.4	8.2	ARL/arl	29	2.59	2	6.04	56.5	30.0	58
8/ICA 43.4	8.3	ARL/arl	18	2.24	0	3.11	56	15.0	34
8/ICA 43.4	8.4	ARL/arl	35	2.49	2	10.28	62	37.5	55
8/ICA 43.4	8.6	ARL/arl	16	1.90	1	6.06	63.5	20.0	48
12/ICA 7.2.12	12.3 12.5	ARL/arl	31	2.90	2	6.44	51.5	27.5	74
12/ICA 7.2.12	12.4	ARL/arl	44	3.19	2	8.45	51.5	30.0	78
12/ICA 7.2.12	12.6	ARL/arl	26	2.83	2	4.82	50	15.0	68
16/ICA7.2.10	16.2	ARL/arl	52	3.30	0	1.83	52	10.0	22
18/ICA 7.2.4	18.2 18.7	ARL/arl	4	1.18	0	3.98	51	10.0	18
18/ICA 7.2.4	18.3 18.6	ARL/arl	27	2.60	0	6.74	55	30.0	34
18/ICA 7.2.4 -	18.4,5	ARL/arl	24	2.71	3	4.31	51	17.5	46
18/ICA 7.2.4	18.7	ARL/arl	68	3.67	7	12.28	50	47.5	66
20/ICA7.2.1	7.2/20.2,.3	ARL/arl	13	2.14	0	2.07	52	5.0	19

[z] Number of seeds with greater than five perforations. [y] Susceptibility Index.

Appendix 5.9 continued

Backcross	Line/Vial No.	Arcelin allele present	Adult Emergence (no.)	SI[y]	Severity[z]	WT loss (%)	DAE 50%	Perforated seeds (%.)	EGGS (no.)
20/ICA7.2.1	7.2/20.4	ARL/arl	25	2.80	1	7.33	50	22.5	69
27/ICA 7.3.1	27.1	ARL/arl	7	1.80	0	2.09	47	10.0	18
27/ICA 7.3.1	27.3	ARL/arl	13	1.66	0	4.04	67	20.0	28
27/ICA 7.3.1	27.1, 27.3	ARL/arl	27	2.45	3	6.70	58.5	20.0	27
22/ICA 43.6	22.1	ARL/arl	112	3.76	9	20.81	54.5	60.5	148
22/ICA 43.6	22.3	ARL/arl	164	4.98	16	23.15	44.5	60.0	167
22/ICA 43.6	22.4	ARL/arl	19	2.81	2	4.06	45.5	20.0	29
22/ICA 43.6	22.5	ARL/arl	18	2.30	1	10.80	54.5	20.0	29
22/ICA 43.6	22.7	ARL/arl	31	2.57	3	0.94	58	21.6	15
228/ICA 43.4	228.2	ARL/arl	25	2.88	1	4.30	48.5	32.5	38
132/BT1.2.4	132/'05-1	ARL/arl	24	2.53	1	2.58	54.5	37.5	39
132/BT1.2.4	132/'05-2	ARL/arl	25	2.50	0	5.27	56	35.0	23
231/ICA43.6	231.2	ARL/arl	54	3.07	8	10.34	56.5	35.0	53
231/ICA43.6	231.3	ARL/arl	23	2.35	2	5.32	58	15.0	43
231/ICA43.6	231.4	ARL/arl	32	2.60	3	7.25	58	32.5	50
231/ICA43.6	231.5	ARL/arl	31	2.79	1	6.34	53.5	40.0	15
213/ICA 37.1	213.1	ARL/arl	21	2.70	1	6.69	49	22.5	41
213/ICA 37.1	213.2	ARL/arl	28	2.95	0	5.42	49	40.0	28
222/ICA38.1	222.6	ARL/arl	36	2.83	4	7.63	55	22.5	18
222/ICA38.1	222.7	ARL/arl	17	2.80	1	2.95	44	10.0	69
222/ICA38.1	222.6,.7	ARL/arl	3	1.02	0	0.96	47	2.5	17
ICA29/7.3/04	29.1	ARL/arl	51	3.28	3	15.11	52	42.5	97
ICA223/43.3	223.2	ARL/arl	37	3.37	1	10.06	46.5	40.0	32
ICA29/7.3/04	29.2	ARL/arl	24	2.65	0	6.85	52	25.0	35
183/5-593	183/5-593	ARL/arl	29	2.73	1	9.13	53.5	27.5	32
16/ICA7.2.10	16.2,5,6	ARL/arl	44	2.69	3	8.00	61	35.0	65

[z] Number of seeds with greater than five perforations. [y] Susceptibility Index.

Appendix 5.9 continued

Backcross	Line/Vial No.	Arcelin allele present	Adult Emergence (no.)	SI[y]	Severity[z]	WT loss (%)	DAE 50%	Perforated seeds (%.)	EGGS (no.)
191/ICA	191.1	ARL/arl	10	2.08	0	6.54	48	20.0	45
193/ICA 12.1	193.1	ARL/arl	28	2.78	3	9.77	52	15.0	26
193/ICA 12.1	193.2	ARL/arl	20	2.66	0	5.33	49	25.0	25
193/ICA 12.1	193.3	ARL/arl	15	2.38	1	4.73	49.5	20.0	19
186/ICA6.2	186.2	arl/arl	150	5.00	17	32.67	43.5	73.3	112
186/ICA6.2	186.3	arl/arl	123	4.70	12	37.02	44.5	50.0	98
191/ICA	191.2	arl/arl	65	4.12	6	15.93	44	45.0	52
191/ICA	191.3	arl/arl	139	4.98	16	33.39	43	76.7	89
195/ICA 12.2	195.1	arl/arl	39	3.66	5	10.12	43.5	22.5	48
195/ICA 12.2	195.3	arl/arl	65	3.98	5	14.66	45.5	42.5	47
59/ICA -16	59.1	arl/arl	46	3.78	3	12.97	44	62.5	46
59/ICA -16	59.2	arl/arl	87	4.22	6	17.76	46	67.5	72
59/ICA -16	59.3	arl/arl	126	4.77	14	30.52	44	60.0	108
45/ICA28.2	45.3	arl/arl	50	3.54	4	12.95	48	37.5	42
45/ICA28.2	45.2	arl/arl	151	5.01	16	32.71	43.5	63.3	98
202/ICA 16.1	202.1	arl/arl	117	4.31	12	23.25	48	62.5	76
202/ICA 16.1	202.2	arl/arl	99	4.11	10	20.22	48.5	55.0	47
202/ICA 16.1	202.3	arl/arl	40	3.20	3	10.12	50	27.5	36
52/ICA20.1	52.1	arl/arl	34	3.09	2	7.45	49.5	30.0	20
52/ICA20.1	52.2	arl/arl	22	2.68	1	7.28	50	30.0	28
52/ICA20.1	52.3	arl/arl	31	3.01	2	6.66	49.5	32.5	27
15/ICA 43.5	15.1	arl/arl	27	3.01	1	6.74	47.5	30.0	38
15/ICA 43.5	15.2	arl/arl	87	4.17	6	18.12	46.5	57.5	74
15/ICA 43.5	15.3	arl/arl	28	2.98	2	6.56	48.5	25.0	28
20/ICA7.3	20/7.3-1	arl/arl	28	3.29	2	6.33	44	25.0	25
20/ICA7.3	20/7.3-2	arl/arl	67	4.01	5	12.23	45.5	45.0	56

[z]Number of seeds with greater than five perforations. [y] Susceptibility Index.

Appendix 5.9 continued

Backcross	Line/Vial No.	Arcelin allele present	Adult Emergence (no.)	SI[y]	Severity[z]	WT loss (%)	DAE 50%	Perforated seeds (%.)	EGGS (no.)
20/ICA7.3	20/7.3-3	arl/arl	56	3.93	4	12.63	44.5	45.0	50
6/ICA7.2.16	6.5	arl/arl	20	2.99	3	4.15	43.5	27.5	20
ICA Pijao	ICA Pij1	arl/arl	53	3.92	8	23.20	44	63.3	82
ICA Pijao	ICA Pij2	arl/arl	35	3.36	6	25.38	46	43.3	46
ICA Pijao	ICA Pij3	arl/arl	52	3.99	7	20.20	43	46.7	67
ICA Pijao	ICA Pij4	arl/arl	86	4.40	7	23.02	44	56.7	75

[z]Number of seeds with greater than five perforations. [y] Susceptibility Index