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Identification of potential seed storage protein responsible for bruchid resistance in common bean landraces from Tanzania and Malawi

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Bean bruchids are among the most devastating insect pests of common bean that can inflict huge losses in storage. To identify potential resistance to these pests, screening was performed at Sokoine University of Agriculture. Two resistant landraces were identified, viz Kalubungula and KK25. Recombinant inbred (RI) KSy, KSw and ML populations were created from crosses between Soya × Kalubungula, Soworo × Kalubungula and Nagaga × KK25, respectively. Seed storage proteins were characterized and sequenced in RI population progenies to determine if phenotypic resistance was associated with α-amylase inhibitor – phytohemagglutinin – arcelin (APA) storage proteins. We found no association between the seed storage proteins observed in Kalubungula and its recombinant inbred lines with an APA protein. KK25 and its progenies had Arcelin-5, Leucoagglutinin, Erythroagglutinin and a hypothetical seed storage protein that conditions antibiosis effects as a resistance mechanism. The hypothetical seed storage protein observed in these lines may contribute to enhanced resistance.

Key words: α-amylase, phytohemagglutinin, arcelin, *Acanthoscelides obtectus*, *Zabrotes subfasciatus*, common beans.

INTRODUCTION

Common bean (*Phaseolus vulgaris* L.) is the principal grain legume grown as a major source of protein as well as an important source of income to many farmers in developing countries (Broughton et al., 2003). Common bean is mostly cultivated by small scale farmers who cannot afford the technologies that would enhance their ability to grow and securely store their crop. Consequently, they store the grains on-farm under open conditions where they incur a wide range of postharvest losses including insect pest infestations (Cardona et al., 2005). The most serious storage pests of beans are the bean seed weevils. Two species, namely *Acanthoscelides obtectus* and *Zabrotes subfasciatus* are the major species infesting beans in Tanzania. Their distribution is

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temperature dependent, with Z. *subfasciatus* being confined to warmer areas and *A. obtectus* being confined to cooler areas (Blair et al., 2010). Most of small-scale farmers use pesticidal plants and other indigenous methods for bruchid control of small quantities of seeds (Mbongo et al., 2013; Mutungi et al., 2020; Kusolwa et al., 2013). These methods are less efficient in most cases due to poor availability of pesticidal plants. One of the promising ways of controlling them is using host plant resistance.

Legume seeds contain different compounds that are essential for embryo and seed development as well as for defense against insect pests. Among these compounds are tannins, cyanogenic glucosides, non-protein amino acids and proteins such as protease, α-amylase inhibitor, lectins, chitinases, β-1, 3-glucanases, phaseolin and arcelins. Some of these compounds have nutritional value and others are antinutritional with antibiosis effects against both vertebrate and invertebrate seed consumers (Baldin et al., 2017; Sales et al., 2000). In common bean, the important seed storage proteins that have been identified and characterized are phaseolin, lectins, trypsin inhibitor and lectin-like proteins (Gepts and Bliss, 1988; Lioi et al., 2003).

Phaseolin is the major storage protein in common bean that account for 50% of total seed storage protein and provides essential amino acids to seed consumers (Bollini and Chrispeels, 1978). Lectins and lectin-like proteins are the anti-nutritional seed storage proteins that defend bean seeds against insect pests. The lectin and lectin-like proteins include phytohemagglutinin, arcelins and α-amylase inhibitors. α-amylase inhibitors are a lectin-like proteins that acts as pesticides in bean due to their ability to prevent carbohydrate digestion. These proteins also possess chitinolytic activity by hydrolysing chitinous exoskeletons as well as internal perithropic gut membranes of insect pests (Dayler et al., 2005). Arcelins are also lectin-like proteins made up of polypeptides that are closely related to phytohemagglutinins and α-amylase inhibitors but possess different intrinsic specificities for complex sugars that make it toxic to insect pests (Minney et al., 1990).

Generally, phytohemagglutinin and α-amylase inhibitor are present in wild and cultivated genotypes of common bean while arcelins are found only in wild genotypes of common bean (Sparvoli, et al., 2001). Different arcelin alleles have been introduced from wild common bean as well as tepary bean (*P. acutifolius*) into experimental lines, and some of these lines have been deployed to breeding programs in Africa (Tigist et al., 2021). Accession G40199 of tepary bean is among the wild accessions found to confer high level of resistance to bruchid infestation (Kusolwa and Myers, 2012). A previous survey by Sokoine University Agriculture found two landraces (Kalubungula from Tanzania and KK25 from Malawi) to be resistant to bruchids. The mechanisms of resistance and possible storage proteins related to resistance in these landraces are unknown. This study focused on characterizing and investigating the seed storage proteins related to bruchid resistance in KK25, Kalubungula and derived progenies from KK25 and Kalubungula crosses with susceptible parents.

MATERIALS AND METHODS

Study area

The study was conducted at Sokoine University Agriculture (SUA) and Oregon State University (OSU). Seed was multiplied at SUA whereby after crossing parents and producing F_1 seeds, F_2 seeds were advanced to F_3 generation. After harvest and drying, the grains were stored at -20ºC for two days in order to eliminate any field acquired bruchid infestations. The F_3 seeds were then taken to OSU for laboratory analysis of seed storage proteins.

Plant materials

The bean landraces used in this study included bruchid resistant, red-seeded 'Kalubungula' and 'KK25' from Tanzania and Malawi respectively collected by Bean Bruchid Resistance Project supported by McKnight Foundation at SUA. The susceptible landraces used in crosses were two farmers' preferred varieties 'Soya' and 'Soworo' from Tanzania and crossed to Kalubungula, and 'Nagaga' from Malawi and crossed to KK25. These landraces were part of the major bean collection from farmers' saved seed in major bean growing regions in Tanzania and Chitedze Agriculture Research Station (CARS) in Malawi. The Soya × Kalubungula cross was designated KSy while the Soworo × Kalubungula cross was designated KSw. From the 101 $F_{2:3}$ families obtained, 53 genotypes were from Soya × Kalubungula and 48 from Soworo × Kalubungula. Nagaga × KK25 population was generated by Kananji (2007) and consisted of 3 genotypes in the F_3 generation. These bean genotypes together with the Tanzanian lines were used in protein profiling and sequencing.

Protein extraction

The samples were prepared as described by Osborn et al. (1986) with some modification. Cotyledons of individual seeds were scraped on sandpaper to obtain a fine powder. Ten milligrams (10 mg) of the cotyledon flour of each seed were placed in the microfuge tube and suspended in 200 µl of extraction solution (0.5 M NaCl, pH 2.4), shaken vigorously and vortexed. The mixture was allowed to settle at room temperature for 30 min and centrifuged at 11200 x g for 2 min. Thereafter, 3 µl of the supernatant was mixed in a microfuge tube with 3µl of 0.5 M NaCl pH 2.4 and 6 µl of 2x protein-based sample buffer from BIORAD (65.8 mM tris HCl pH 6.8, 26.3% [w/v] glycerol, 2.1% SDS, 0.5% 2-mercarptoethanol, 0.01% Bromophenol blue). The mixtures were transferred to the Polymerase Chain Reaction (PCR) plates and heated for 5 min in a thermocycler at 94°C to denature the tertiary protein structures into primary structures. Ten microliter (10 µl) of each sample was immediately loaded onto a 15% pre-cast Tris-glycine SDS-PAGE running gel (BIORAD) and was electrophoresed at 200 V constant for 50 min in 1x Laemmli SDS-PAGE running buffer (25 mM Tris-base, 192 mM glycine, 0.1% w/v SDS, pH 8.3). The gels were placed in a sealable plastic container with 100 ml of staining

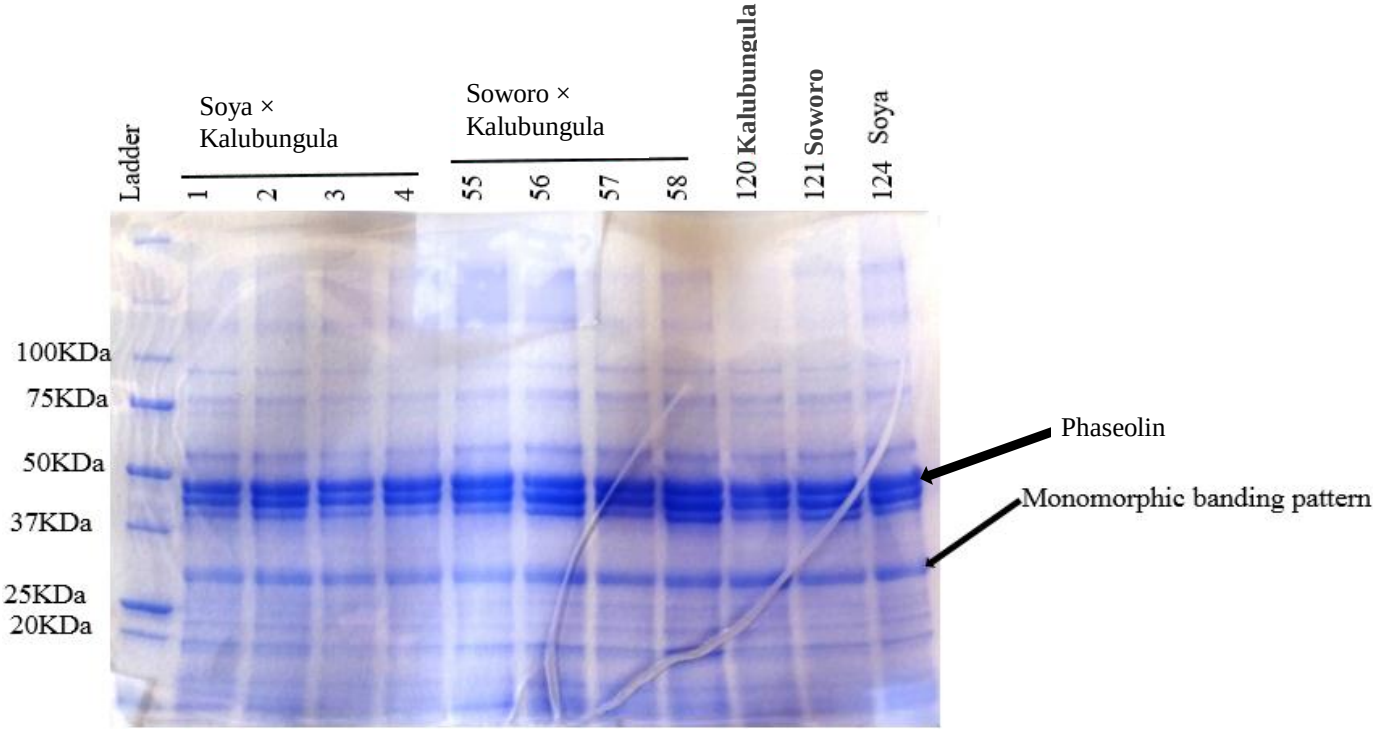

Figure 1. Bean seed storage profile separated on 15% SDS-PAGE gel with an arrow indicating a 33kDa monomorphic banding pattern between parents and progenies. Protein ladder molecular weight on left, lanes 1-6 are Kalubungula × Soya RILs, 55-58 are Kalubungula × Soworo RILs, 120 is Kalubungula, 121 is Soworo and 124 is Soya

solution (40% methanol, 10% acetic acid, 0.1% Coomassie brilliant blue R-250®). They were agitated for 1-2 h on a platform shaker at low speed and destained (40% methanol, 10% acetic acid) overnight. The gels were then washed three times and shaken gently in deionized water for 15 min then placed between pre-wetted cellophane (BIORAD) to dry. The gels were scored with reference to a 33kDa protein subunit based on electrophoretic mobility of size standard proteins. Gels were photographed to retain permanent documentation.

Protein isolation from SDS-PAGE gels and sequencing

We isolated and sequenced protein as described by Kusolwa (2007) with little modification. Unique bands from the gels were excised with a sterile scalpel and cut into 1-mm pieces then placed in microfuge tubes. The gel plugs were washed twice, whereby 200 µl of deionized water were added, soaked for 15 min, vortexed occasionally and centrifuged for 5 min. The liquid was then removed by a pipette after each spin. The gel plugs were washed 2 times to remove Coomassie brilliant blue stain. We added 200 µl of a 50% of acetonitrile mixed with 50% of 50 mM NH_4HCO_3 solution, soaked for 30 min, occasionally vortexed and centrifuged for 5 min. The liquid was then removed by a pipette. To dehydrate the gel plugs, we added 500 µl acetonitrile and left the mixtures to stand, vortexed occasionally until they turned opaque, centrifuged for 5 min, and removed the liquid. The plugs were dried for 30 min in a vacuum centrifuge. Thereafter the plugs were rehydrated by adding 25 mM NH_4HCO_3 containing 20 ng/µl trypsin pH 8.0, chilled on ice for 45 min. Buffer was added to ensure thorough rehydration of the plugs followed by trypsin digestion for six hours in the dark at 37°C. The supernatant was extracted to new microfuge tubes, and the gel plugs were extracted 3 times whereby 50 µl of 50% acetonitrile were added; thereafter the mixtures were vortexed briefly and centrifuged for 5 min. The supernatant was combined in a new centrifuge tube. The samples were submitted to Mass Spectrophotometry Laboratory (MS-MS Lab.) for sequencing at OSU.

RESULTS

Protein profiles

The total seed storage protein from cotyledons of the Tanzanian and Malawian landraces and their progenies was profiled by one-dimension SDS-PAGE gels. There were no polymorphic bands of seed storage proteins observed in Tanzanian landraces, progenies, and susceptible checks. Instead, a monomorphic band was observed in the region of 33kDa, where arcelins and α-amylase inhibitors typically are found (Figure 1). This same band was present in Malawian RILs, but a second polymorphic band was observed at approximately 26kDa in Nagaga x KK25 progenies (Figure 2). This band was present in the KK25 landrace as well but absent from the other Malawian landrace (Nagaga) as well as all

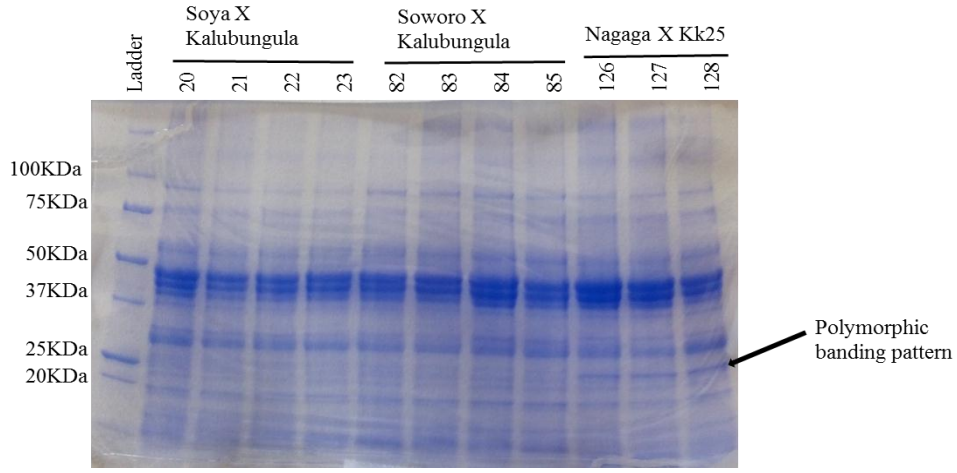

Figure 2. Bean seed storage proteins separated on 15% SDS-PAGE gel. Protein ladder molecular weight in first lane, 20-23 are Soya × Kalubungula RILs, 82-85 are Kalubungula X Soworo RILs, 126-127 are Nagaga X KK25 RILs. An arrow indicates the unique ~26kDa band observed.

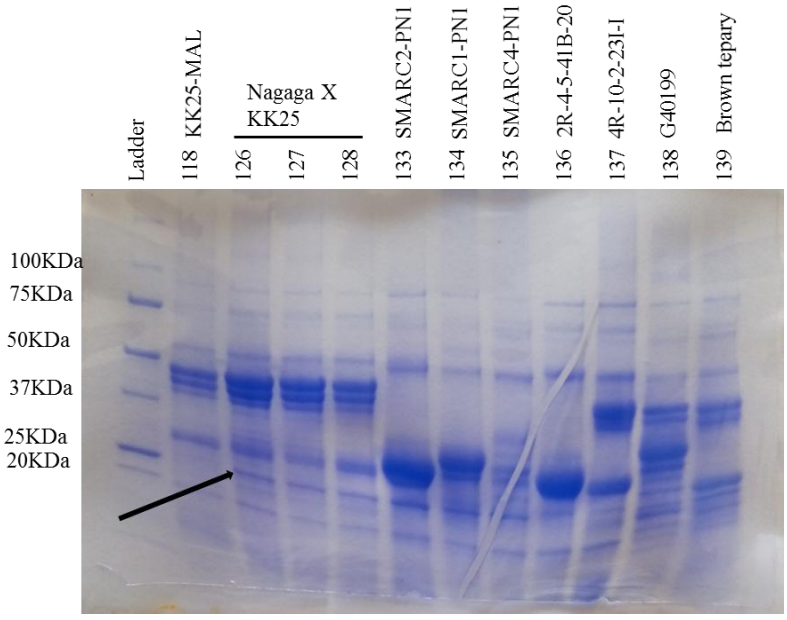

Figure 3. Bean seed storage profile separated on 15% SDS-PAGE gel. Protein ladder molecular weight on left, 118 is KK25- MAL, 126-127 are Nagaga/KK25 RILs, and 133-139 are the arcelin-2, arcelin-1 and arcelin-4, phaseolin null SMARC lines. The last four lanes (136 – 139) show seed storage protein patterns derived from tepary bean and introgressed into common bean (136 – 137) as well as two tepary parents (138 – 139). Arrow points to a band found in KK25 and Nagaga/KK25 RILs that is at a similar location to the arcelin containing SMARC lines.

Tanzanian landraces and progenies. Arcelin-containing bean lines used as bruchid resistant checks were compared for banding pattern with KK25 and progeny, and the banding pattern at 26kDa was similar for the SMARC lines but not the tepary and tepary-derived lines (Figure 3). Both Kalubungula, Nagaga and KK25 and

Table 1. Amino acid sequences produced from 26 kDa protein fragments of Nagaga X KK25 RILs with their matching proteins from NCBI database.

Protein size (kDa)	Observed	Peptide sequence	Match sequence	Matched protein	Reference
		ML3(Nagaga x KK25)			
26kDa	25.58kDa	YTDDMELDDAVHTAILTLKEGFEGQISGK	1	ARC-5	Hamelryck et al. (1996)
	26.35kDa	HSLLGASGEISDFQEILRYLDELILYDNMWDDGNSLGPK	6	PHA-e	Nagae et al. (2016)
	26.35kDa	FNPLWNALVLGGVK	3	PHA-l	Hamelryck et al., 1996
		ML10 (Nagaga x KK25)			
26kDa	26.77kDa	ATFLGEIITSLPTLGAGQSAFK	1	ARC-5	Hamelryck et al. (1996)
26kDa	26.77kDa	IYDYDVYDNLGDPDK	1	PHA-l	Chrispeels and Raikhel. (1991)
26kDa	26.77kDa	LDSQVYGDHTSQITK	-	Hypothetical *Phaseolus vulgaris* protein	This work

their progenies were of the Andean Center of Domestication based on the triplet banding pattern of phaseolin (Figures 1 and 2) located between 37-50kDa.

Amino acid sequencing

Sequencing of the excised protein bands at 26kDa from Nagaga × KK25 recombinant inbred lines revealed the presence of both arcelin and phytohemagglutinin. One of the amino acid sequences from ML10 (indicated by 127 in Figures 2 and 3) did not correspond to any previously reported proteins in the genus *Phaseolus* but appears to be an uncharacterized or hypothetical *Phaseolus vulgaris* protein. The observed amino acid sequences and their corresponding protein matches from NCBI are shown in Table 1.

BLAST search of the observed amino acid (aa) (Figure 4) sequences revealed that the sequences were 100% identical to Phytohemagglutinin sequence of the *P. vulgaris* accession 101A and 1FAT; and with above 96.0% aa identity and similarity to CAJ34351, CAD28838, CAD28674, respectively. Two sequences from ML3 (Nagaga x KK25) also matched sequence to leucoagglutinin with matching ranging from one to three matches. One sequence resembled the erythroagglutinin type of phytohemagglutinin with six sequence matches (Figures 5 and 6). Leucoagglutinin (PHA-l) (Figure 4) from Tanzania genotypes had above 96.4% similarity score with 1FAT-A, CAJ34351, CAD28838, CAD286774 PHA-l from *P. vulgaris*, and distantly similar to *P. acutifolius and P. costaricensis* by 93.1 and 92.3% respectively (Supplementary Figures 1 and 2). The erythroagglutinin (PHA-e) sequence from ML3 (Figure 5) had higher amino acid identity with Erythroagglutinating phytohemagglutinin sequences from GenBank, including 5AVA_A, XP_007152771, P05088, AHB17899, and CAD28837 with 100, 99.6, 98.9, 98.2 and 97.8%, respectively (Supplementary Figures 3 and 4). The observed similarity indicate there is little variation between the observed amino acid sequences compared to the reference protein sequence in NCBI while the sequence matching to arcelin-5 from ML3 and ML10 (Figure 6) had 'aa' identity of 100, 99.6 and 96.2% with sequences of 1IOA_a, Q42460.2, and Q41116.1, respectively, from GenBank (Supplementary Figures 5 and 6).

DISCUSSION

Protein characterization from KSy and KSw populations showed the presence of a monomorphic band at approximately 33kDa. This band that was also observed in susceptible bean genotypes checks had a relatively similar molecular size like arcelins-like proteins. However, the banding pattern was different from that typical of the APA proteins. This and the fact that both susceptible and resistant parents had the band indicated that the near-33kDa storage proteins in these bean lines was not likely related to resistance to bruchids. Kananji (2007) identified bean landraces (KK35, KK73 and KK90) that lacked arcelins but exhibited resistance. In an

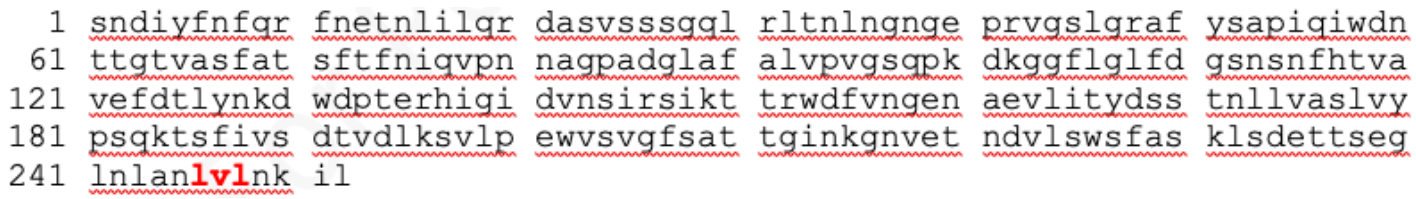

Figure 4. Amino acid sequences of the 25kDa protein band from ML3 and ML10, the Nagaga × KK25 RIL's matched to leucoagglutinin (PHA-l) of *Phaseolus vulgaris.* Matched sequences are shown in red colour and bold.

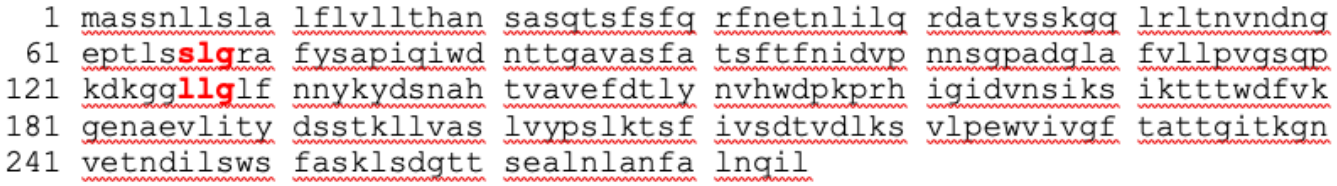

Figure 5. Amino acid sequences of the 25KDa protein band from ML3 and ML10, the Nagaga × KK25 RIL's matched to erythroagglutinin (PHA-e) of *Phaseolus vulgaris.* Matched sequences are shown in red colour and bold.

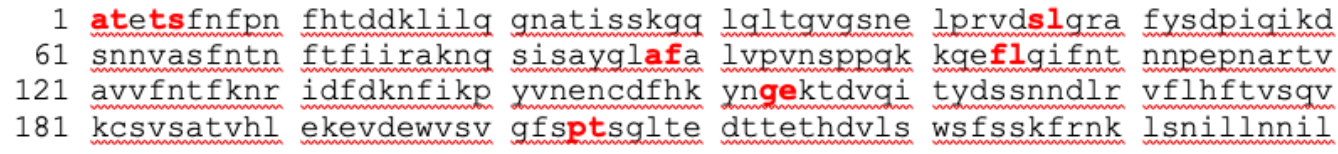

Figure 6. Amino acid sequences of the 25KDa protein band from ML3 and ML10, the Nagaga × KK25 RIL's matched to arcelin-5 of *Phaseolus vulgaris.* Matched sequences are shown in red color and bold.

experiment with those lines, increased numbers of *A. obtectus* adult bruchids emerged when the seed coats were removed, suggesting that resistance was conferred by the seed coat. He concluded that the seed coat acted as a physical and/or chemical barrier to attacks by bruchids.

Sales et al. (2000) found that the presence of vicillins and legumins in the seed coat of broad bean *Vicia faba* deterred development of the first instar larvae of cowpea weevils *Callosobruchus maculatus*. Silva et al. (2004) provided supporting evidence that vicillins or phaseolin present in the seed coat of *P. vulgaris* were detrimental to *C. maculatus* development. They found that the seed coat thickness was not important but high vicillins concentration in the seed coat was an important factor for resistance. Lattanzio et al. (2005) reported that high concentration of tannins in undamaged seeds of cowpea conferred a biochemical defense that deterred, poisoned, or starved bruchid larvae. We believe that the seed coat confers resistance in the Tanzanian Kalubungula landrace and its progenies, but further studies are needed to confirm this assertion.

Presence of seed storage proteins at 26kDa in Nagaga × KK25 and its recombinant inbred lines suggested that resistance was storage protein based. Amino acid sequencing from the trypsin digested protein fragments from the 26kDa band revealed the presence of trace amount of protein peptides corresponding to arcelin-5, like that observed by Hamelryck et al. (1996), phytohemagglutinin-l observed by Chrispeels and Raikhel (1991) and phytohemagglutinin-e of *P. vulgaris* similarly observed by Nagae et al. (2016). These protein peptides have a special property of binding glycan in a complex structure of a back-fold conformation which affects activities of glycosyltransferases enzymes and localization of carrier glycoproteins in an insect. These seed storage proteins are known to defend common bean against bruchids by interacting with the glycoprotein, interfering with carbohydrate digestion, and binding to the intestinal cells of insect. It is possible that the mechanism of

resistance in these Malawian landraces is antibiosis conferred by presence of an arcelin/ phytohemagglutinin-like protein though other factors might be involved. Kusolwa and Myers (2012) observed the presence of multiple variants of the antibiosis seed storage proteins of the complex APA locus in progenies of crosses between wild tepary bean (*Phaseolus acutifolius*) accession G40199 highly resistant to bean bruchids and a susceptible common bean cultivar (ICA Pijao). Our protein peptide sequencing demonstrated low and weak sequence matching (1-6 match) with reference proteins in the databank, which suggests that the protein observed in KK25 and progeny may be quite novel and different from previously characterized proteins of similar size. This novel storage protein may not be the only source of resistance in these line and other factors may have contributed to the observed resistance. The presence of an uncharacterized sequence in one of the KK25 progeny may be of importance to breeders and may contribute to new knowledge about seed storage proteins and bruchid resistance.

Conclusion

Intriguing is the lack of seed storage proteins conferring bruchid resistance in the Tanzanian lines. We recommend that the role of the seed coat and its potential to mitigate bruchid damage be assessed. Further investigation is needed to determine if the unique 26kDa proteins are responsible for bruchid resistance in Malawian lines or if there are other factors involved. Further studies are needed to better define the storage proteins observed in Malawian lines.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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SUPPLEMENTARY FIGURES

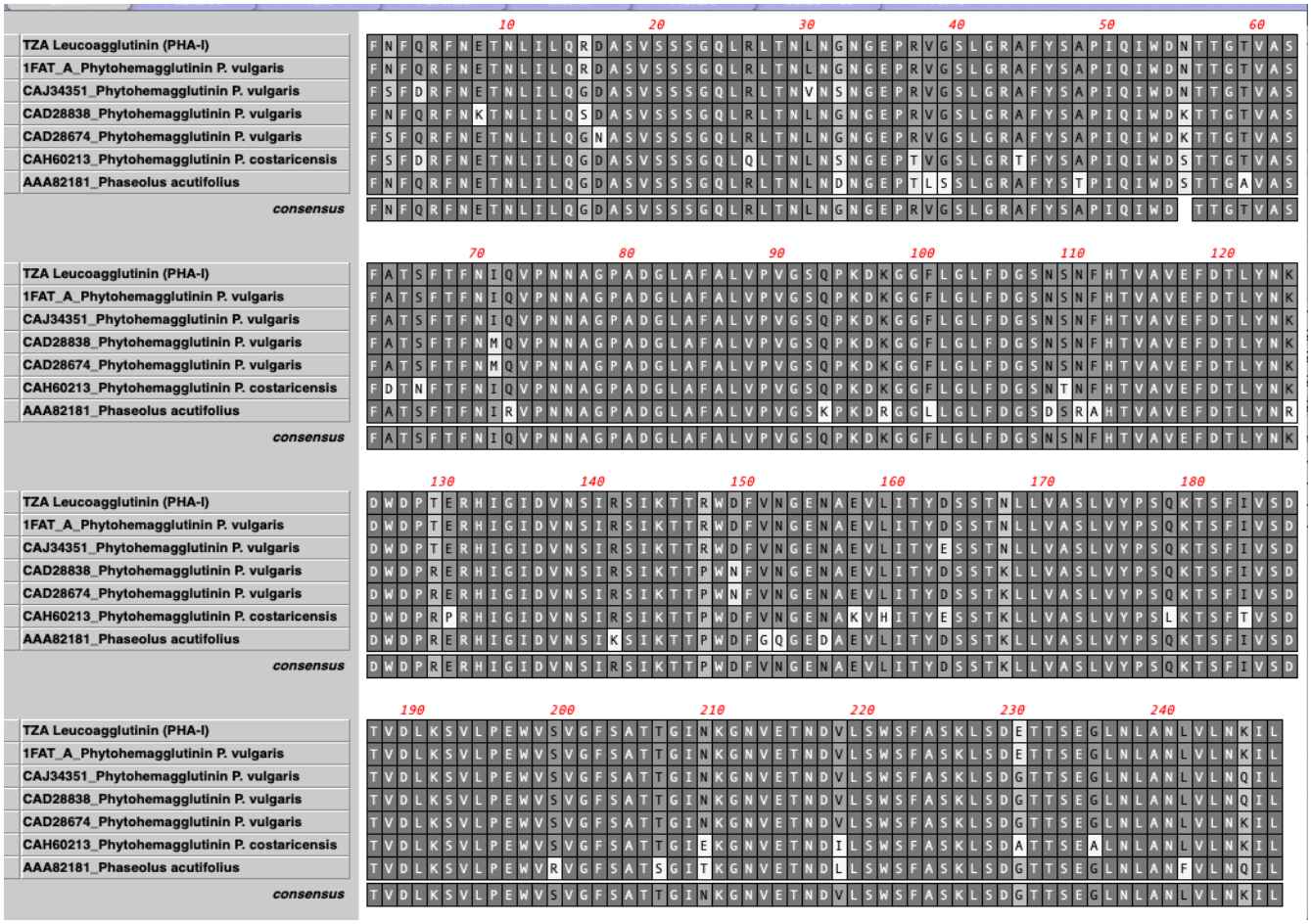


Figure 1. Amino acid alignment of TZA Phytohemagglutinin leucoagglutinin (PHA-l) with GenBank deposited Phytohemagglutinin sequences.

```
                                                                ** Identity Scores (%) **
                                                TZA Leu  1FAT_A_  CAJ3435  CAD2883  CAD2867  CAH6021  AAA8218
                                                coagglu  Phytohe  1_Phyto  8_Phyto  4_Phyto  3_Phyto  1_Phase
                                                tinin (  magglut  hemaggl  hemaggl  hemaggl  hemaggl  olus ac
                                                PHA-l)   inin P.  utinin  utinin   utinin   utinin   utifoli
                                                          vulgar  P. vulg  P. vulg  P. vulg  P. cost  us
TZA Leucoagglutinin (PHA-l)                       100.0    100.0     96.8     96.0     96.0     90.3     87.9
1FAT_A_Phytohemagglutinin P. vulgaris             100.0    100.0     96.8     96.0     96.0     90.3     87.9
CAJ34351_Phytohemagglutinin P. vulgaris            98.0     98.0    100.0     94.7     95.1     91.5     87.4
CAD28838_Phytohemagglutinin P. vulgaris            96.8     96.8     96.0    100.0     98.0     89.9     88.7
CAD28674_Phytohemagglutinin P. vulgaris            96.4     96.4     96.8     98.4    100.0     91.1     88.3
CAH60213_Phytohemagglutinin P. costaricensis       92.3     92.3     93.9     92.7     93.5    100.0     84.6
AAA82181_Phaseolus acutifolius                     93.1     93.1     93.1     93.9     93.9     91.1    100.0
                                                ** Similarity Scores (%) **

**Similarity Scores(s) are shown below the diagonal (x) with Identity Scores(I) above**
  a b c d e
a x i i i i
b s x i i i
c s s x i i
d s s s x i
e s s s s x
```

Figure 2. Similarity matrix of TZA Phytohemagglutinin leucoagglutinin (PHA-l) sequences with GenBank deposited Phytohemagglutinin sequences.

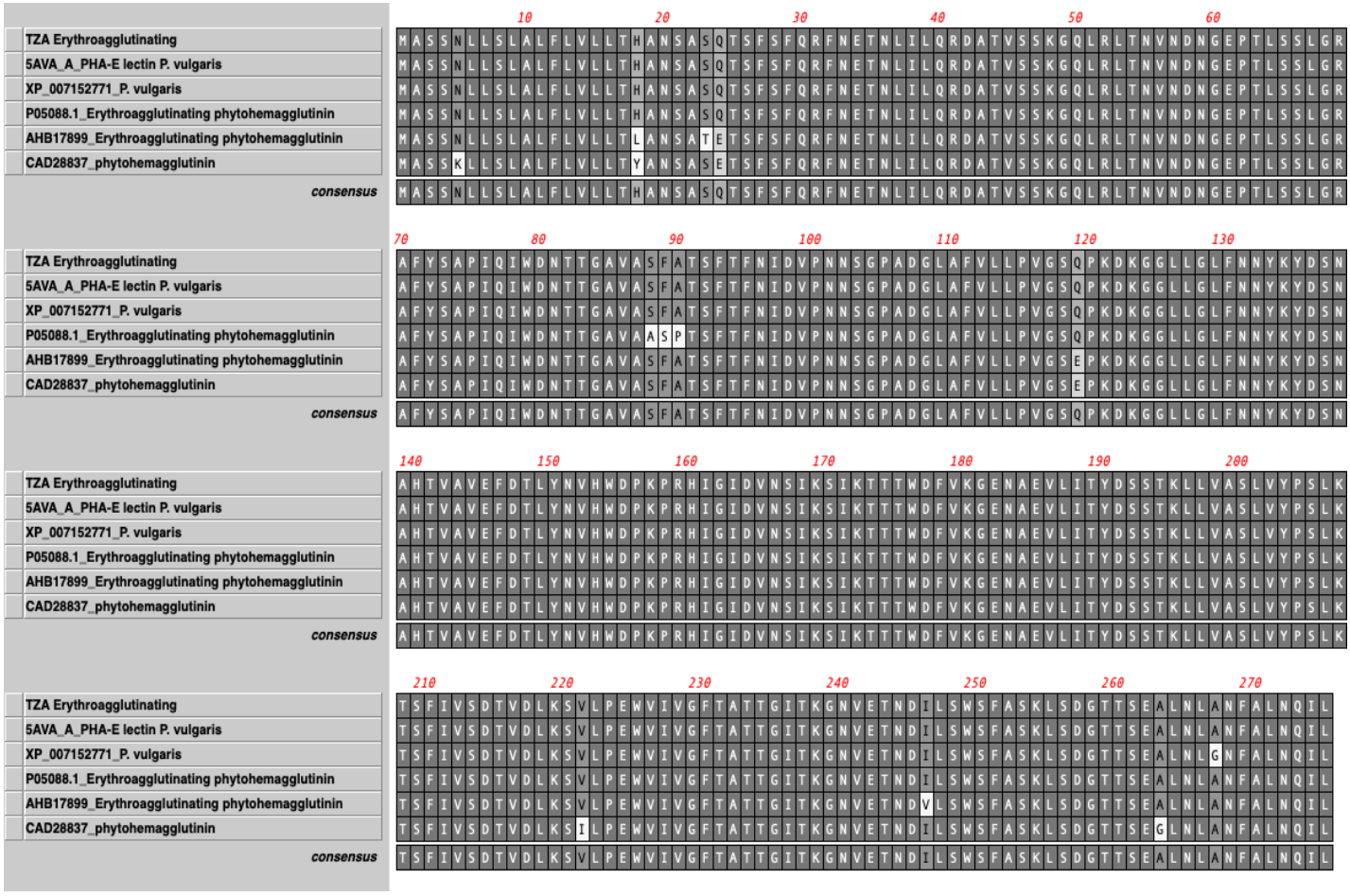
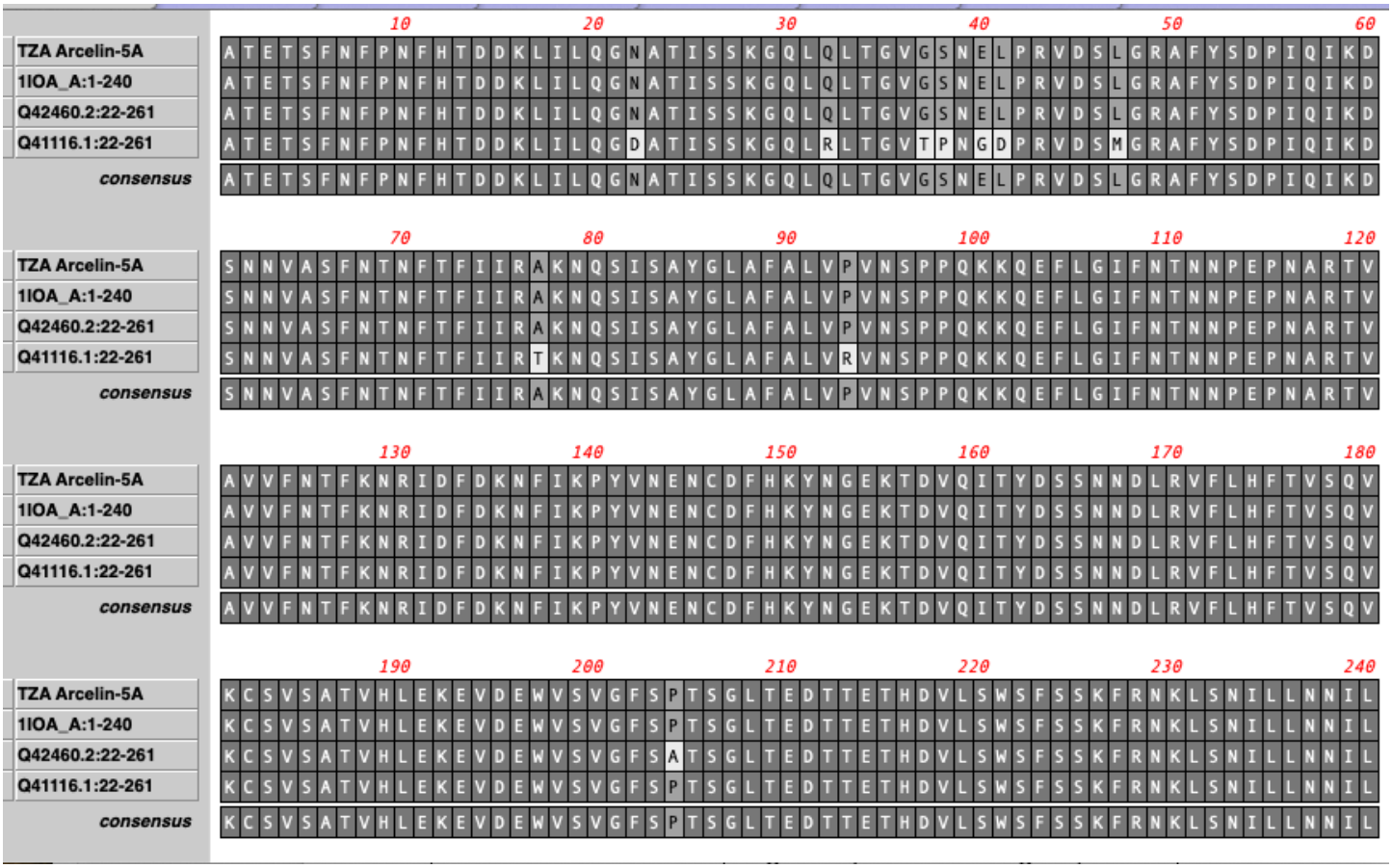


Figure 3. Amino acid alignment of TZA Phytohemagglutinin erythroagglutinin (PHA-e) with GenBank deposited erythroagglutinin sequences.

```
                                                    ** Identity Scores (%) **
                                                TZA Ery  5AVA_A_  XP_0071  P05088.  AHB1789  CAD2883
                                                throagg  PHA-E l  52771_P  1_Eryth  9_Eryth  7_phyto
                                                lutinat  ectin P  . vulga  roagglu  roagglu  hemaggl
                                                ing (PH  . vulga  ris      tinatin  tinatin  utinin
                                                A-e)     ris              g phyto  g phyto
TZA Erythroagglutinating (PHA-e)                 100.0    100.0     99.6     98.9     98.2     97.8
5AVA_A_PHA-E lectin P. vulgaris                  100.0    100.0     99.6     98.9     98.2     97.8
XP_007152771_P. vulgaris                          99.6     99.6    100.0     98.5     97.8     97.5
P05088.1_Erythroagglutinating phytohemagglutinin  99.3     99.3     98.9    100.0     97.1     96.7
AHB17899_Erythroagglutinating phytohemagglutinin  99.6     99.6     99.3     98.9    100.0     97.8
CAD28837_phytohemagglutinin                       98.9     98.9     98.5     98.2     98.9    100.0
                                               ** Similarity Scores (%) **

**Similarity Scores(s) are shown below the diagonal (x) with Identity Scores(I) above**
  a b c d e
a x i i i i
b s x i i i
c s s x i i
d s s s x i
e s s s s x
```

Figure 4. Similarity matrix of TZA Phytohemagglutinin erythroagglutinin (PHA-e) sequences with GenBank deposited erythroagglutinin sequences.



Supplementary Figure 5. Amino acid alignment of TZA Arcelin-5A with GenBank deposited Arcelin sequences.

```
                    ** Identity Scores (%) **
                 TZA Arc   1IOA_A:   Q42460.   Q41116.
                 elin-5A   1-240     2:22-26   1:22-26
                                     1         1
TZA Arcelin-5A     100.0     100.0     99.6      96.2
1IOA_A:1-240       100.0     100.0     99.6      96.2
Q42460.2:22-261     99.6      99.6    100.0      95.8
Q41116.1:22-261     97.5      97.5     97.1     100.0
               ** Similarity Scores (%) **

**Similarity Scores(s) are shown below the diagonal (x) with Identity Scores(I) above**
  a b c d e
a x i i i i
b s x i i i
c s s x i i
d s s s x i
e s s s s x
```

Supplementary Figure 6. Similarity matrix of TZA Arcelin-5A sequences with GenBank deposited Arcelin sequences.