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Altering protein composition by genetically removing phaseolin from common bean seeds containing arcelin or phytohemagglutinin

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Abstract Arcelin seed proteins of common bean (*Phaseolus vulgaris* L.) confer resistance to bruchid pests, and in vitro results suggested that greater resistance could be achieved by increasing the concentration of arcelin. We created backcross lines having arcelin alleles (SMARC lines), or alleles for the related protein phytohemagglutinin (PHA) (SMPHA lines), and a null allele for phaseolin to determine if seeds lacking phaseolin would contain increased quantities of arcelin or PHA proteins. To test the affects of genetically removing phaseolin, SMARC and SMPHA lines were derived as pairs of phaseolin-containing and phaseolin-null lines. Parental, SMARC, and SMPHA lines were grown in a replicated greenhouse trial and measured for days-to-flower, days-to-maturity, seed weight, and for quantities of phaseolin, arcelin dimer, PHA, and total proteins. There were no differences between pairs of phaseolin and phaseolin-null lines for days-to-flower, seed weight or total protein, and inconsistent differences for days-to-maturity. Arcelin concentrations were significantly increased in two of four pairs of SMARC lines, and PHA concentration was significantly greater in four of five pairs of SMPHA lines. These or other changes in the seed protein composition in phaseolin null lines may improve resistance to bruchids.

Key words *Phaseolus vulgaris* · Seed proteins · PHA · Protein compensation · Insect resistance

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Introduction

Infestation of the common bean (*Phaseolus vulgaris* L.) by the Mexican bean weevil, *Zabrotes subfasciatus* (Boheman), and the bean weevil, *Acanthoscelides obtectus* (Say), causes worldwide loses of 7–35% annually, and these loses are particularly detrimental where beans are an important source of dietary protein (Labeyrie 1981). Wild bean accessions with high levels of resistance to *Z. subfasciatus* and *A. obtectus* have been identified (Schoonhoven et al. 1983) which contained four allelic variants of a novel seed protein, arcelin (arc-1, arc-2, arc-3, and arc-4; Osborn et al. 1986). In artificial seeds, higher levels of resistance to *Z. subfasciatus* were obtained with higher concentrations of purified arcelin proteins (Osborn et al. 1988a). Backcross lines and artificial seeds containing some arcelin variants were highly resistant to *Z. subfasciatus* (Osborn et al. 1988a; Cardona et al. 1990), but the concentration of arcelin in backcross lines was not effective against *A. obtectus* (Osborn et al. 1988a).

Our aim was to increase the concentration of arcelin proteins by genetically manipulating the quantity of other seed-specific proteins. The two major seed proteins of common bean cultivars are phaseolin and phytohemagglutinin (PHA), which make up 40–60% and 6–12%, respectively, of the total seed protein (Osborn 1988). In Sanilac backcross lines, arc-1 can contribute approximately 50% of the total protein (Osborn et al. 1988a). Each of these major protein fractions is controlled by a single Mendelian gene with alleles for the presence of different variants co-dominant to each other (phaseolin and PHA, Brown et al. 1981a; arcelin, Osborn et al. 1986; Kornegay et al. 1993) and dominant to the lack of expression conditioned by null alleles (phaseolin, Gepts and Bliss 1984; PHA, Brown et al. 1981b; arcelin, Osborn et al. 1986). Despite the fact that these proteins account for the majority of total protein, the absence of any or all of these proteins

is compensated by the remaining protein fractions so that the total concentration of protein within the seed either remains the same or increases (Osborn and Bliss 1985; Confalonieri et al. 1988; Delaney and Bliss 1991; Burow et al. 1993).

To test whether genetically removing phaseolin would increase the concentrations of arcelin and phytohemagglutinin proteins, we developed backcross lines in a Sanilac background which contained phaseolin (PP lines), or the null allele for phaseolin (PN), in combination with alleles for either the arc-1, -2 or -4 variants (SMARC lines) or PHA variants from 'Bunsi,' 'Protop P-1,' or 'Viva' (SMPHA lines). The SMPHA lines were developed because arcelin and PHA are related members of the phytohemagglutinin-arcelin-α-analyse inhibitor gene family which is believed to play an important role in plant defense against pests (Osborn et al. 1988a; Chrispeels and Raikhel 1991; Hartweck et al. 1991; Mirkov et al. 1994). The concentrations of arcelin, phaseolin, and the different PHA variant proteins in these lines were determined and the paired PN and PP lines were compared.

Materials and methods

Development of lines

Experimental lines were developed in a 'Sanilac' background by backcrossing with selection for different protein compositions (Fig. 1). Two or three lines were derived from each initial cross. The female parent in the initial crosses was experimental line MB11-29 (Burow et al. 1993) which contained null alleles for arcelin, PHA, and phaseolin (genotype designation *arl/arl*, *lec/lec*, *phs/phs*, Bassett 1989) and is estimated to consist of 72% 'Sanilac' germplasm. The male parents in the initial crosses were common bean lines SARC1, SARC2 and SARC4 which contain arcelin variant proteins arc-1, arc-2 or arc-4, respectively, in a background of approximately 87.5% Sanilac (Osborn et al. 1988a; Hartweck et al. 1991), or the cultivars Bunsi, Protop-P1, and Viva which contain the PHA protein variants, PHA-B, PHA-P and PHA-V, respectively.

After crossing the F_1 seed to L12-56 (87.5% Sanilac background), BC_1 seeds were screened for the presence of arcelin and/or PHA and phaseolin protein. Arcelin and phaseolin were detected by Ouchterlony double-diffusion assays (Ouchterlony 1967). Polyclonal antibodies were raised against arc-1, -2 or -4 as described previously (Hartweck et al. 1991). Hemagglutination assays were used to test for the presence of PHA as described previously (Hartweck et al. 1991). Hemagglutination assays also were used in screening for the presence or absence of arcelin in some lines because of tight linkage between the genes for arcelin and PHA (Osborn et al. 1986). Agglutination-positive lines were later checked for the presence of arcelin by the Ouchterlony method.

BC_1 seeds containing arcelin, PHA, and phaseolin (*Arl/arl*, *Lec/lec*, *Phs/-*) were planted and, based on progeny testing, a single BC_1 plant with the genotype *Arl/arl*, *Lec/lec*, *Phs/phs* was chosen as the sole source for the further derivation of lines within the set. The progeny of these selected BC_1 plants segregated into 27 genotypes but only three of these genotypes were desired (*Arl/Arl*, *Lec/Lec*, *Phs/Phs*; *Arl/Arl*, *Lec/Lec*, *phs/phs* and *arl/arl*, *lec/lec*, *phs/phs*). Seeds with correct phenotypes were planted and the desired genotypes were chosen by progeny testing. The three genotypes were then selfed again and the BC_1S_3 seed grown for evaluation. Lines containing phaseolin were designated PP (phaseolin present) and lines containing the phaseolin null allele designated PN (phaseolin null). The double-null lines were designated N-PN (null for arcelin and/or PHA and null for phaseolin). The final SMARC and SMPHA lines contained approximately 83.6% and 61.8% Sanilac germplasm, respectively.

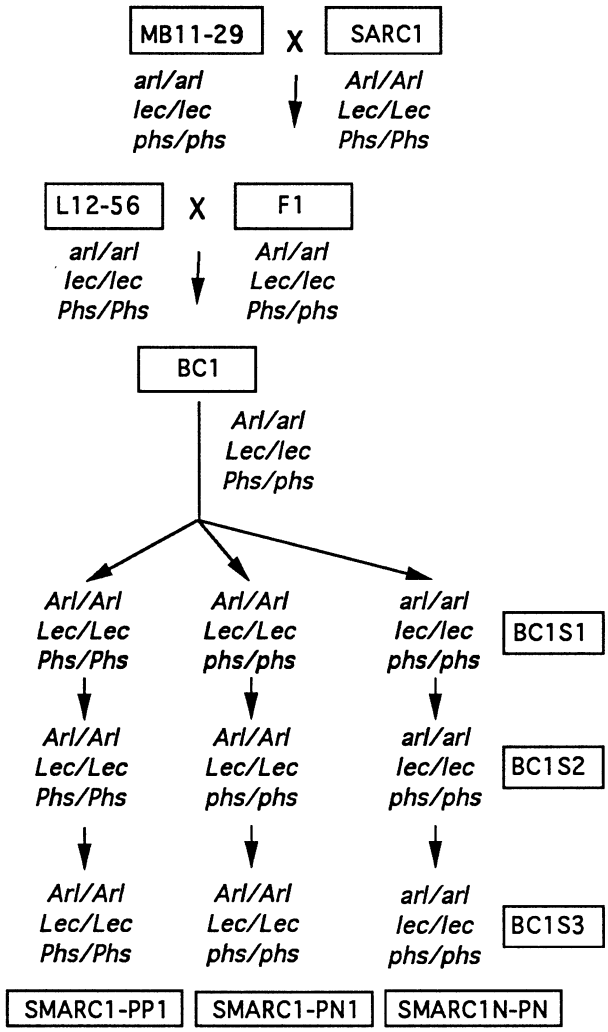

Fig. 1 Breeding scheme used for developing one set of SMARC1 lines. The origins of parental lines are described in Materials and methods. Genotypic symbols for arcelin, PHA, and phaseolin are *Arl*, *Lec*, and *Phs*, respectively (Bassett 1989). Parental lines MB11-29 and SARC1 were crossed to produce an F_1 which was backcrossed to L12-56 (both SARC1 and L12-56 contain 87.5% Sanilac germplasm). The BC_1 had eight genotypes but only *Arl/arl*, *Lec/lec*,*Phs/-* seeds were planted and *Arl/arl*,*Lec/lec*,*Phs/phs* plants were selected based on progeny tests. The BC_1S_1 had 27 genotypes, but only three genotypes were selected based on BC_1S_1 seeds and their progenies. A second set of *PP* (containing phaseolin) and *PN* (null for phaseolin) lines (designated PP2 and PN2) and two sets of SMARC and three sets of SMPHA lines containing other variants of arcelin or PHA were derived by the same scheme

Each initial cross (MB11-29 × arcelin/PHA donor) was repeated once using different male and female plants from within the parental populations. This resulted in the development of a second pair of independently derived lines for each protein variant. For example, the first SMARC1 pair were named SMARC1-PP1 and SMARC1-PN1 and the second independently derived pair were named

SMARC1-PP2 and SMARC1-PN2. The double null lines (N-PN) were not derived with the second pair.

The experimental lines were grown in a replicated greenhouse trial and measured for several traits. Each experimental line and the parental lines MB11-29, L12-56 and Sanilac were replicated four times in the greenhouse. One experimental unit consisted of five plants which were grown separately in 18-cm-diameter clay pots with sufficient spacing to prevent competition for light.

Seed characteristics

Days-to-flowering (DTF) was measured as days-from-planting to when at least three plants within an experimental unit had begun to flower. Days-to-maturity (DTM) was measured as days-from-planting to the stage when at least three plants had dry pods. The weight of 50 randomly drawn seeds (SWT) was also measured. Seeds were cleaned, dried at 55°C, and ground in a Wiley mill using a 30-mesh sieve. Nitrogen determinations were made by combustion of dry bean flour using a Leco FP-428 Nitrogen Determinator System (Leco Corporation, St. Joseph, Michigan) and EDTA was used in calibration. Total protein concentration was estimated as percentage nitrogen $\times 6.25$.

The quantities of phaseolin, PHA and arcelin for each experimental unit were determined by rocket gel-imunoelectrphoresis as previously described (Weeke 1973; Osborn et al. 1985) except that Tris-Barbitol buffer was used as recommended by the manufacturer (Sigma). Phaseolin, arc-2 dimer, and PHA-B, PHA-P and Sanilac PHA were purified (Sun et al. 1978; Hartweck et al. 1991) and used in antibody production as described previously (Osborn et al. 1988b).

Crude seed proteins were extracted from 0.1 g of bean flour in 5 ml of extraction buffer (0.5 M NaCl 0.01% NaN_3) to optimize the solubilities of all proteins. A portion of the extraction was carbamylated by mixing 1 : 1 with 2 N KOCN and heating to 50°C for 20 min (Weeke 1973). The samples were then handled as previously described (Osborn et al. 1985). Purified standard proteins were dissolved in extraction buffer and protein concentrations were determined based on comparison to known concentrations of acetylated BSA (Promega) in extraction buffer using the microbicinchoninic colorimetric assay (Smith et al. 1985; Pierce Biochemicals). Standard proteins were carbamylated after quantification.

The quantity of phaseolin protein in crude extracts was determined by incorporating phaseolin antibody into the agarose gel and comparing the height of rockets formed by the crude sample extracts to those of a dilution series of phaseolin standard proteins (0.72, 1.65, 2.07, 2.78, and 3.11 mg/ml) run on the same plate. For the quantification of arc-1 and arc-2 dimer proteins, the crude extracts were first diluted 1 : 4 in extraction buffer and then carbamylated. The concentration of standard arc-2 dimer proteins was determined (0.68, 1.90, 3.39, 5.22, and 8.80 mg/ml) and then diluted 1 : 4 in extraction buffer and carbamylated. Arc-2 dimer antibody was used to quantify both arc-2 dimer and arc-1 dimer protein, because the proteins are approximately 99% similar (John and Long 1990) and appear immunologically identical using tandem-crossed immunoelectrophoresis (Kroll 1973) (data not shown). There were no arcelin dimer proteins in the arc-4-containing lines identified by Western blotting (Hartweck et al. 1991) or the Ouchterlony tests (data not shown). The quantities of PHA in lines containing PHA-B or PHA-V were determined by incorporating antibody made against PHA-B into the agarose gel and comparing the height of rockets to those of a dilution series of pure PHA-B protein (0.06, 0.13, 0.25, 0.59, and 1.33 mg/ml). This was feasible because all of the polypeptides in PHA-V are also found in PHA-B (Brown et al. 1982) and there were no differences in antigenic reactions based on Ouchterlony assays (data not shown). PHA-P was quantified using antibody made against PHA-P and standardized to pure PHA-P protein (0.01, 0.12, 0.32, 0.63, and 1.32 mg/ml). For the parental line, Sanilac, antibody produced against purified Sanilac PHA was incorporated into the gel and purified Sanilac PHA protein was used as the standard (0.11, 0.21, 0.49, 1.05, and 1.95 mg/ml).

Statistical analyses

Analyses of variance were performed using the general linear model procedure of SAS (SAS Institute Inc. 1982). Due to differences in variation of the measured traits, the SMARC and SMPHA lines were analyzed separately. For comparison of PN and PP paired lines, the model used in the analysis of variance was a nested design (Damon and Harvey 1987) with protein variant type (arc-1, -2, and -4, or PHA-B, -P, and -V) as the main factor and phaseolin type (PN and PP) as a nested factor. Parental lines MB11-29, Sanilac, L12-56 and double-null lines were not included in the comparisons of paired lines. Both protein-variant and phaseolin-type effects were considered fixed. The phaseolin-type error term was used to estimate the variance for *t*-tests between PN and PP means with a correction for the unequal number of replications where necessary. A randomized complete block design was used to calculate LSDs for comparisons between all SMARC or SMPHA lines and parents.

Results

Seed characteristics

Arcelin dimer, PHA, and phaseolin were quantified using the results from rocket gel-electrophoresis (Fig. 2). Arc-1 tetramer and arc-4 tetramer proteins were not quantified because they produced complex patterns in rocket gels against antibodies made using a mixture of the proteins. The low levels of PHA proteins in the SMARC lines were also not quantified. No phaseolin was detected in PN or N-PN lines (Fig. 2).

There was little variation among SMARC lines for total protein concentration (Table 1). In the analysis of variance, the presence versus absence of phaseolin was not a significant factor for total protein, and there were no significant differences between pairs of PP and PN lines. All SMARC1 and SMARC2 lines contained more arcelin dimer protein than phaseolin (Table 1). Although the presence versus absence of phaseolin was not a significant factor in the nested analysis of variance for arcelin-dimer concentration, in paired *t*-tests, two of the four pairs of lines contained significantly more dimer protein in PN lines than PP lines (Table 1).

The SMPHA-PP lines contained greater quantities of phaseolin than the SMARC-PP lines, although total protein levels were similar (Table 2). The presence versus absence of phaseolin was not a significant factor in the analysis of variance for total protein concentration in SMPHA lines, but was a significant factor for PHA concentration. In four of five comparisons between paired lines, there were significantly higher concentrations of PHA in PN lines than in PP lines (Table 2).

Neither the protein-variant type nor the presence or absence of phaseolin had significant effects on DTF (data not shown) or DTM, over all SMARC and SMPHA lines, but there were significant differences for DTM between specific pairs of PP and PN lines. The

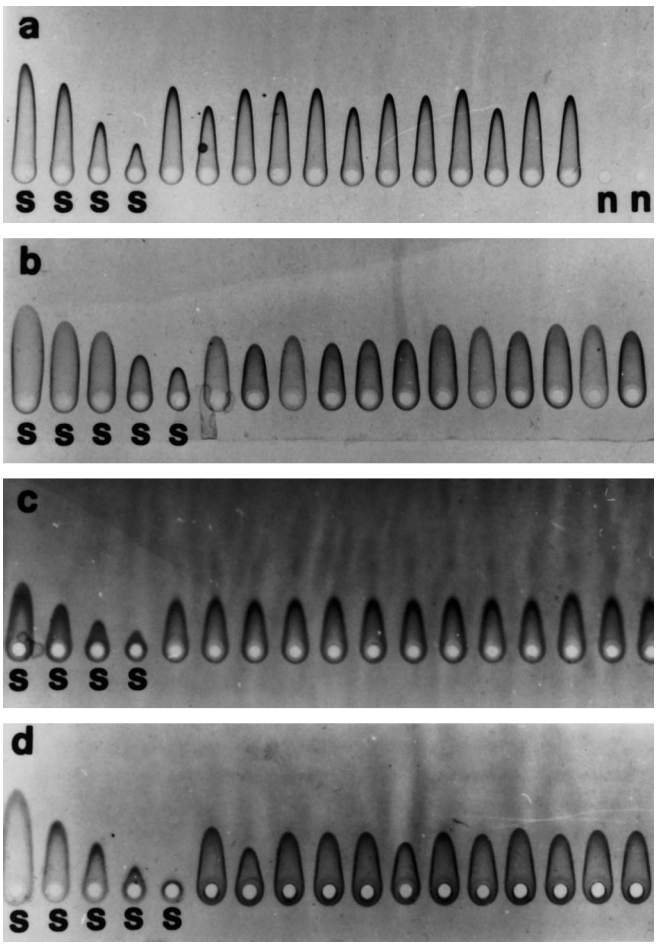

Fig. 2a–d Examples of rocket gels used to quantify seed proteins. In gels containing phaseolin antibody (panel **a**), pure phaseolin standards (labelled *s*) were used to quantify the phaseolin concentration in SMARC-PP (unlabelled) and SMARC-PN (labelled *n*) lines. In gels containing arcelin-2 antibody (panel **b**), arcelin-2 protein standards (*s*) were used to quantify the concentration of dimer protein in SMARC1 and SMARC2 lines (unlabelled). In gels containing PHA-B antibody (panel **c**), PHA-B standards (*s*) were used to quantify the PHA protein in SMPHAB and SMPHAV lines and in Bunsi and Viva (unlabelled). In gels containing PHA-P antibody (panel **d**), PHA-P standards (*s*) were used to quantify PHA-P protein in SMPHAP lines and Protop (unlabelled)

SMARC2-PN lines matured 15 days later than paired SMARC2-PP lines (Table 1), and SMPHAV-PP2 matured later than SMPHAV-PN2 (Table 2). These factors also had no overall significant effects on seed weight in either the SMARC or SMPHA lines, although significant differences in both directions were observed for some pairs of PP and PN lines (Tables 1 and 2).

Discussion

The objective of this study was to genetically increase the concentration of arcelin or PHA proteins in common bean seeds. Pairs of lines either with or without phaseolin protein were derived and compared to determine if the lack of phaseolin protein would be compensated by a concomitant increase in arcelin or PHA proteins. All lines had a similar level of total protein, indicating that the lack of phaseolin was compensated for by increases in other protein fractions. In some of the newly created lines, arcelin or PHA concentrations were increased. Other studies have reported protein compensation in the reverse orientation, with phaseolin compensating for the lack of arcelin (Romero Andreas et al. 1986) or PHA (Osborn and Bliss 1985). Soybean 11*s* proteins can also compensate for low levels of 7*s* globulin proteins (Ogawa et al. 1989).

In measurements of related seed characteristics, the paired lines showed no significant differences for DTF, and inconsistent differences of SWT. For DTM, there were differences between the SMARC and SMPHA lines. The SMARC-PN lines were either later maturing (SMARC2) or had values which were not significantly

Table 1 Means and least-significant differences for concentrations of phaseolin and arcelin-dimer proteins determined by rocket gel-immunoelectrophoresis, concentration of total protein determined by nitrogen analysis, days-to-maturity (DTM), and seed weight (SWT) in SMARC lines

Line	Protein fraction (mg/100 mg flour)			DTM	SWT (g/50 seeds)
	Phas.	Dimer	Total		
SMARC1-PN1	0.00	13.64	24.26	94.5	9.5
SMARC1-PP1	3.98	14.88	22.54	94.0	9.7
SMARC1-PN2	0.00	***17.19***[a]	23.81	96.3	10.1
SMARC1-PP2	1.61	***12.23***	23.20	96.0	9.9
SARC1	ND[b]	10.84	22.38	99.8	9.6
SMARC1N-PN1	0.00	0.00	22.44	86.0	11.1
SMARC2-PN1	0.00	16.79	26.33	***105.3***	11.3
SMARC2-PP1	4.62	15.26	24.72	***88.0***	9.2
SMARC2-PN2	0.00	***14.97***	25.53	***105.0***	10.6
SMARC2-PP2	3.43	***10.83***	23.73	***92.0***	10.7
SARC2	3.47	13.55	24.63	94.5	ND
SMARC2N-PN1	0.00	0.00	22.69	88.0	ND
SMARC4-PN1	0.00	ND[b]	24.44	94.0	***11.0***
SMARC4-PP1	4.25	ND	24.23	97.8	***9.6***
SMARC4-PN2	0.00	ND	23.06	93.0	10.1
SMARC4-PP2	3.44	ND	24.71	96.0	10.6
SARC4	3.37	ND	25.44	97.0	7.5
SMARC4N-PN1	0.00	ND	23.44	107.0	ND
MB11-29	0.00	ND	24.91	ND	ND
Sanilac	6.30	ND	24.88	87.1	11.20
L12-56	9.37	ND	25.42	87.1	11.20
LSD[c]	0.47	1.02	1.56	26.0	1.36

[a] Comparison between pairs of PN and PP lines were performed by a *t*-test using the mean square error from the phaseolin-type factor of the nested ANOVA described in Materials and methods. Pairs which were significantly different are in bold italics

[b] ND – not determined

[c] Least-significant differences were calculated for all lines using the mean square error from the complete block design ($P = 0.05$)

Table 2 Means and least-significant differences for concentrations of phaseolin and PHA proteins determined by rocket gel-immunoelectrophoresis, concentration of total protein determined by nitrogen analysis, days-to-maturity (DTM), and seed weight (SWT) in SMPHA lines

Line	Protein fraction (mg/100 mg flour)			DTM	SWT (g/50 seeds)
	Phas.	PHA	Total		
SMPHAB-PN1[a]	0.00	***7.99***[b]	27.44	86.5	9.8
SMPHAB-PP1	7.36	***3.89***	23.96	90.0	11.2
Bunsi	8.20	4.33	25.88	95.3	10.5
SMPHABN-PN1	0.00	0.00	24.31	94.0	14.9
SMPHAP-PN1	0.00	***6.46***	24.91	95.0	***10.6***
SMPHAP-PP1	6.96	***5.05***	26.04	93.0	***13.3***
SMPHAP-PN2	0.00	4.47	27.04	94.7	13.8
SMPHAP-PP2	8.10	4.51	26.97	94.0	12.7
Protop	8.99	4.69	32.38	97.0	13.1
SMPHAPN-PN1	0.00	0.00	24.56	89.0	9.9
SMPHAV-PN1	0.00	***6.24***	24.89	94.8	12.0
SMPHAV-PP1	8.65	***4.64***	24.46	101.3	13.4
SMPHAV-PN2	0.00	***6.34***	25.23	***90.0***	***12.1***
SMPHAV-PP2	7.57	***3.16***	24.34	***98.0***	***10.0***
Viva	6.80	3.30	21.79	96.0	12.8
SMPHAVN-PN1	0.00	0.00	24.69	97.3	11.7
LSD[c]	0.53	0.39	2.80	17.5	2.5

[a] The second set of SMPHAB lines were lost in germination
[b] and [c] See Table 1 for explanations

different from the PP lines. In SMPHA lines, the effect was the opposite: PN lines matured earlier (SMPHAV) or were not significantly different from the SMPHA-PP lines. These maturity differences might be explained by differences in the accumulation rates of proteins needed for the seeds to reach final total protein levels.

These lines have not been evaluated for nutritional quality. However, another arcelin-1 backcross line, RAZ-2 (Cardona et al. 1990), was examined for anti-nutritional affects in rat-feeding trials (Putzai et al. 1993). The raw flour of RAZ-2 was significantly less toxic than the raw flour of the parental cultivar without arcelin, and all toxicity was abolished by cooking (Putzai et al. 1993). The PN lines may have other nutritional differences related to the lack of phaseolin protein and increased levels of compensating proteins. Changes in phaseolin can effect changes in methionine levels (Gepts and Bliss 1984; Burrow et al. 1993), an amino acid which is important for nutrition and found in limiting quantities in common bean (Bressani 1973). The nutritional and anti-nutritional properties of lines with altered protein levels should be evaluated before cultivar release.

Seed-protein genetic mutants can be powerful tools in the design of new cultivars with altered protein levels. Among the paired lines we developed, the absence of phaseolin was accompanied by significant increases in arcelin-dimer quantity for two out of four paired SMARC lines and in PHA quantity for four out of five SMPHA lines. Increased levels of arcelin, PHA and/or other proteins compensating for the lack of phaseolin may affect the biology of bruchid pests. Results from testing these backcross lines for bruchid resistance are reported in a companion paper.

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