Generation of a Mutant Population for TILLING Common Bean Genotype BAT 93

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ABSTRACT. Common bean (*Phaseolus vulgaris*) is the major food legume worldwide, making it an important target for novel approaches of genetic analysis. This study evaluated the use of ethyl methane sulfonate (EMS) for the generation of a mutant population for targeted induced local lesions in genomes (TILLING) in common bean. TILLING is a powerful reverse genetics approach that uses a large mutant population for identification of mutants in loci of interest. Based on overall survival, development, and yield of treated seed, 40 mM EMS was found to be an appropriate concentration for the generation of a mutant population in common bean genotype BAT 93. Higher concentrations of EMS resulted in survival rates of less than 10% and lower concentrations resulted in the generation of fewer mutants. Based on TILLING results from other species, a population of 5000 lines is estimated to be sufficient for saturation of the common bean genome. Phenotypic mutation frequencies and the isolation of targeted mutations in the BAT 93 mutant population indicate that mutagenesis was effective.

Common bean is the principal grain legume used for direct human consumption worldwide (Broughton et al., 2003) and is increasingly recognized for its high nutritional quality. Thus, it is an important target for intensive genomics research such as reverse genetic approaches like targeted induced local lesions in genomes (TILLING). Common bean is a true diploid with 11 chromosomes and a haploid complement of 588 (Bennett and Leitch, 2005) to 637 Mbp (Arumuganathan and Earle, 1991). Its small genome, with a low incidence of duplications, makes it suitable for sequencing and genomic applications and as a reference for the elucidation of genomes of more complex legume species such as soybean (Gepts et al., 2005). *Phaseolus vulgaris* is closely related to other cultivated *Phaseolus* species, including *P. acutifolius* (teary bean), *P. coccineus* (scarlet runner bean), and *P. lunatus* (lima bean) as well as to legumes in the Phaseoleae tribe, including *Cajanus cajan* (pigeonpea),

Glycine max (soybean), *Vigna radiata* (mungbean), and *Vigna unguiculata* (cowpea) (Broughton et al., 2003). Common bean has a well-developed genetic map (Freyre et al., 1998), markers linked to agronomically important traits (Kelly et al., 2003), active development of expressed sequence tag resources (Melotto et al., 2005; Ramirez et al., 2005), and several bacterial artificial chromosome (BAC) libraries (Kami et al., 2006; Vanhouten and MacKenzie, 1999). Although forward genetic tools are rapidly being developed, reverse genetic approaches have been unavailable in common bean as a result of the absence of an effective transformation protocol and thus the inability to develop transposon-based or T DNA-based mutagenesis systems. TILLING has been shown to be effective in legumes such as soybean (Cooper et al., 2008), *Lotus japonicus* (Perry et al., 2003), and *Medicago truncatula* (Javot et al., 2007). The establishment of mutation protocols and mutant populations in common bean is thus important.

Radioisotopes, x-ray, and chemical mutagens have been used previously to induce mutations in common bean. Gamma radiation has been used for the breeding of seed yield and quality (Sarafi, 1973), seed color (Allavena, 1989; Hussein and Disouki, 1976; Wyatt and Dukes, 1980), insect resistance (Mohan et al., 1980), and disease resistance (Allavena, 1989; Tulmann-Neto and Ando, 1976; Zogorcheva and Poriazov, 1983). Gamma radiation has also been used for the study of specific morphological mutations (Frazier and Davis, 1966a, 1966b; Nagata and Bassett, 1984). X-rays were used to modify

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growth habit, resulting in the development of the determinate navy bean cultivar, Sanilac (Andersen and Down, 1956). Several chemical mutagens have also been used, including azide (Cary, 1982), *N*-ethylnitrosourea (Svetleva, 2004), and ethyl methane sulfonate (Motto et al., 1975). Ethyl methane sulfonate (EMS) has been used to generate mutations in seedcoat color (Moh, 1971), biological nitrogen fixation (Davis et al., 1988; Gautam et al., 1998; Pankhurst et al., 2004; Park and Buttery, 1989), phytic acid biosynthesis (Sparvoli et al., 2007), and seed development (Silue et al., 2006).

EMS is the most widely used mutagen in plants for the development of large mutant populations primarily because it creates large numbers of point mutations in almost all species studied, but also because the mutation frequency seems to be independent of genome size (Greene et al., 2003; Henikoff and Comai, 2003; McCallum et al., 2000). Thus, saturation of the genome with mutants can be achieved with relatively small populations. Nevertheless, attempts at TILLING in some species such as rice (*Oryza sativa*) have been confounded by low mutation frequencies and high rates of lethality at higher mutagen concentrations (Till et al., 2007). A balance must therefore be sought between efficient mutagenesis and plant lethality by selecting the highest mutation frequency that yields adequate plant survival. Mutation frequency can be estimated based on phenotypic traits such as albinism and embryo lethality; however, these phenotypic traits can sometimes be difficult to measure (Till et al., 2007; Wu et al., 2005). Previous mutagenesis in common bean using EMS has used concentrations ranging from 10 to 80 mM (Davis et al., 1988; Gautam et al., 1998). In another study, the concentration of EMS, the length of the treatment, and the ambient temperature were found to affect the efficiency of mutagenesis (Pankhurst et al., 2004). Additionally, different species and even different genotypes within the same species can react distinctly to mutagenesis (Till et al., 2007; Wu et al., 2005). Thus, as a result of the variable genotypic response to the mutagen, comprehensive testing of specific genotypes selected for mutagenesis population development is necessary.

Several considerations were evaluated for the selection of a genotype for mutagenesis. The ideal genotype should be broadly adapted; should be well-studied using molecular,

genetic, and physiological methods; should harbor desirable traits for study; and should have high rates of multiplication and ease of agronomic management. To ensure that the mutants of interest are generated, mutations must theoretically be produced at every locus of the genome. Saturation of the *L. japonicus* genome (470 Mb) was achieved through the development of 3800 independent M2 lines (Perry et al., 2003). Because common bean has a larger genome (590 Mb), the appropriate population size to ensure saturation could be larger.

Materials and Methods

PLANT MATERIAL. Common bean genotype BAT 93, a representative of the Mesoamerican gene pool, was bred for high productivity in tropical conditions at the Centro Internacional de Agricultura Tropical (CIAT), Colombia (Voysest, 1983, 2000). It was selected from a double cross population developed between landraces. BAT 93 was selected for mutagenesis (Table 1) because it is one of the parents of the core common bean mapping population (Freyre et al., 1998), it has been used in the generation of a large BAC library (Kami et al., 2006), and it is amenable for production in temperate and tropical environments and under growth chamber, greenhouse, or field conditions (Blair et al., 2005). BAT 93 also possesses several desirable characteristics (Table 1), including disease resistance (Sanchez-Valdez et al., 2004). Seed of BAT 93 were carefully generated for the experiment from single plant selections and confirmed for homogeneity using simple sequence repeat marker analysis at CIAT.

OPTIMIZATION OF EMS CONCENTRATION. Initial experiments on EMS concentrations were conducted as part of the development of a mutagenesis protocol for common bean at the University of Geneva, Geneva, Switzerland. In the first protocol, 40 g of BAT 93 seed (≈ 200 seeds) were added to separate 500-mL flasks and surface-sterilized with 70% (v/v) ethanol for 10 min and 5% (v/v) H_2O_2 for 10 min. After rinsing the seed with sterile water, 180 mL of water containing EMS concentrations ranging from 0 to 80 mM was added to each flask. The flasks were then placed in an incubator shaker and shaken slowly (78 rpm) at 20 °C for 15 h. The seed was then rinsed 20 times with sterile water before planting in trays of

Table 1. Considerations for selecting the common bean BAT 93 genotype for mutagenesis and targeted induced local lesions in genomes (TILLING).

Consideration	Description	Reference
Physiological characterization		
General adaptation	Adapted to temperate and tropical environments	Blair et al., 2005
Plant and seed characteristics	Type III prostrate plant habit, photoperiod insensitive, small beige seeds	Sanchez-Valdez et al., 2004
Disease resistance	Bean common mosaic virus (<i>I</i> gene), rust, angular leaf spot, anthracnose, common bacterial blight	Kelly et al., 2003 (review of mapped QTL); Sanchez-Valdez et al., 2004
Genetic characterization		
Genetic structure of genotype	Homozygous, homogeneous	Blair et al., 2005
Pedigree	(G3709 $\times$ G1320) $\times$ (G3645 $\times$ G5478)	M.W. Blair, personal communication
BAC libraries	Largest common bean bacterial artificial chromosome (BAC) library, 20 $\times$ coverage	Kami et al., 2006; Vanhouten and MacKenzie, 1999
Ongoing EST sequencing efforts	Expressed sequence tag (EST) sequenced from various tissues	Described in Broughton et al., 2003; and in other genotypes (Melotto et al., 2005; Ramirez et al., 2005)

sterile vermiculite (Pankhurst et al., 2004). A second protocol was tested at CIAT (Colombia) and was based on a method used with barley (*Hordeum vulgare*) (Caldwell et al., 2004), the principle difference being the addition of an 8-h imbibition step before EMS treatment. EMS concentrations of 0.0, 19.4, 48.5, 77.7, and 106.0 mM (equivalent to 0%, 0.2%, 0.5%, 0.8%, and 1.0% v/v, respectively; Expt. 1) and 0.0, 19.4, 29.1, 38.8, and 48.5 mM (equivalent to 0%, 0.2%, 0.3%, 0.4%, and 0.5% v/v, respectively; Expt. 2) were tested using this protocol. All seeds were planted in sterilized soil mix and fertilized. Germination data were collected at 7 and 10 d after planting.

After these initial experiments, EMS concentrations were targeted to a narrower range and a larger replicated experiment was conducted at the U.S. Department of Agriculture (USDA), Agriculture Research Service (ARS), Tropical Agriculture Research Station (TARS) in Mayaguez, PR, from Feb. to Apr. 2007. Seeds of BAT 93 were treated with EMS in the laboratory using the first protocol described. Six EMS concentrations, including 0, 20, 30, 40, 50, and 60 mM, were evaluated. The treated seeds were then rinsed 20 times in water and planted in seedling trays in Sunshine Mix #1 (Sun Gro Horticulture, Vancouver, BC, Canada) in a greenhouse. Each EMS treatment was composed of three replicates planted in a

randomized complete block design with the experimental unit being a seedling tray containing 72 seeds. Germination data were collected 7 and 14 d after planting, and plant height data were collected on Day 14. For germination data, the control (0 mM EMS) treatment was adjusted to 100% germination and the other treatments adjusted accordingly. Ten randomly selected surviving seedlings from each replicate were transplanted to 15-cm diameter round pots in Sunshine Mix #1, watered regularly to avoid drought stress, treated with pesticides as needed, and fertilized with Osmocote (14N–6.1P–11.6K; Scotts, Marysville, OH). Seed yield component data were collected at harvest. The experiment was repeated twice. A combined data analysis using both experiments was completed after the homogeneity of error variances was tested. Least significant difference (LSD, $\alpha = 0.05$) was used to compare the mean of each trait by EMS concentration treatment using SAS (SAS Institute, Cary, NC).

MUTANT POPULATION DEVELOPMENT. After mutagenesis of BAT 93 at the University of Geneva (Switzerland), USDA-ARS-TARS (Puerto Rico), and CIAT (Colombia), population development (Fig. 1) was initiated based on protocols developed for *Arabidopsis thaliana* (Colbert et al., 2001; McCallum et al., 2000). Between 2006 and 2008, cycles of

Fig. 1. Protocol developed for the generation of a large mutant population for targeted induced local lesions in genomes (TILLING) in common bean genotype BAT 93 using ethyl methane sulfonate (EMS) seed treatment. Each generation after mutagen treatment is indicated by the letter M followed by the generation number.

mutagenesis were initiated followed by generation advance of manageable subsets of the population. EMS concentrations of 35, 40, 45, and 50 mM were used for the development of the mutagen population. The M1 generation was planted in the greenhouse in Puerto Rico and Colombia and harvested individually at maturity. Eight M2 seeds of each M1 line were planted in the field in Colombia and Puerto Rico during the dry winter season. Leaf material from three to four randomly selected M2 plants was collected and DNA extracted and seed from those same M2 plants was harvested individually. Twenty M3 seeds of each selected M2 line were planted in the field in Puerto Rico and Colombia and M4 seed was bulk-harvested from each line for storage. M4 mutant seed was stored and will be used for conducting both forward (systematic phenotypic screening) and reverse genetic analysis, and M2 DNA will be used for reverse genetic analysis.

PHENOTYPIC EVALUATION OF MUTANT POPULATION. A total of 3000 M2 plants were evaluated for aboveground phenotypic mutations. This population of 3000 M2 plants was generated from 750 independent M1 plants with four M2 plants advanced from each M1 individual. Of the 3000 M2 plants, 844 were generated from a 35 mM EMS treatment and 2156 were generated from EMS concentrations between 40 and 50 mM. If present, mutations were identified in one or more of the M2 plants in the plot of eight plants. The mutations were organized and classified based on mutated tissue, including leaf, stem, seed, and whole plant traits. The percent occurrence of the mutation in the M2 population was then calculated.

For the root nodulation tests, seed from 348 M2 lines, derived from EMS concentrations between 40 and 50 mM, were evaluated. Seeds were surface-sterilized with concentrated H₂SO₄ for 10 min followed by six washes with sterile water and allowed to germinate on B+D agar (Broughton and Dilworth, 1971). Germinated seed were then planted in pots containing sterile vermiculite, watered with the nitrogen-free B+D solution, and inoculated with *Rhizobium etli* strain 1597 before planting. The *R. etli* was grown using standard protocols (Pueppke and Broughton, 1999) and diluted in B+D solution to a final concentration of 10⁷ bacteria/mL for inoculation. After 14 to 18 d, roots of plants with yellow leaves were examined to observe the extent of nodulation. Plants that had no nodules (Nod⁻) or white nodules (Fix⁻) were retained, potted, and grown to maturity.

Results

EFFECT OF EMS CONCENTRATION ON PLANT SURVIVAL, DEVELOPMENT, AND YIELD. Initial experiments testing EMS concentrations on the germination of BAT 93 seed indicated that high concentrations of EMS resulted in a dramatic reduction in germination. In experiments using two different protocols (Fig. 2), concentrations in excess of 40 mM EMS

Fig. 2. Initial experiments on the effects of ethyl methane sulfonate (EMS) concentrations on germination in common bean genotype BAT 93. (A) Percent germination after 7 d using the Pankhurst et al. (2004) protocol. (B–C) Percent germination after 10 d using the Caldwell et al. (2004) protocol. In both protocols, the control (0 mM EMS) treatment was adjusted to 100% germination and the other treatments were adjusted accordingly.

resulted in germination rates of less than 10%, whereas EMS concentrations of 20 mM or lower gave germination rates of 60% or more. Pretreatment of the seed with water for 8 h, using the Caldwell et al. (2004) protocol, resulted in a reduction in overall germination rates (Fig. 2B–C). Based on a target germination rate of 10% or higher, additional EMS treatments were added in the vicinity of the 40 mM treatment in subsequent experiments and the pretreatment of seed with water was eliminated.

Subsequent experiments tested EMS concentrations of 0, 20, 30, 40, 50, and 60 mM and evaluated plant development and seed yield components in addition to germination rates (Table 2). EMS treatment delayed germination as compared with untreated seed. Evaluation of the plants 14 d after germination was found to more accurately reflect actual germination after EMS treatment. Significant differences in germination at 14 d were found between no treatment and EMS concentrations of 40 mM or higher. Although EMS concentrations of 60 mM resulted in germination rates of less than 10%, 50 mM EMS still yielded effective germination rates (greater than 10%). Plant height at 14 d was significantly reduced by EMS treatment and there was little variation in plant height at EMS concentrations above 40 mM.

Seed yield components and other indices, including pod number, seed number, and harvest index, showed a similar response to EMS concentration (Table 2). Significant reductions in pod number occurred with 30 mM EMS or more and in seed yield and harvest index with 20 mM EMS or more. There were no significant differences in these yield traits between EMS treatments above 30 mM. Aboveground vegetative biomass followed an opposite trend to seed yield from 0 to 30 mM EMS, which indicates that there was vegetative yield compensation as a result of reduced seed yield. At higher EMS concentrations (40 to 60 mM), however, vegetative biomass was increasingly affected by the EMS treatment.

EMS-treated seedlings that survived after germination did not necessarily produce seed. Seedlings that survived EMS concentrations of 50 and 60 mM showed less than 50% transplant

survival (Table 2). Overall survival showed an inverse response with increasing EMS concentration. Large reductions in overall survival occurred with concentrations of 30 mM or higher EMS. Concentrations of 50 and 60 mM EMS resulted in overall survival rates of less than 10%.

MUTANT POPULATION DEVELOPMENT. Effective TILLING requires near saturation of the haploid complement of the genome in a large mutant population. Mutation frequency and tolerance to mutation load varies significantly from species to species (Table 3) and may be influenced by ploidy (Comai and Henikoff, 2006; Henikoff and Comai, 2003; Stadler, 1932). Based on the average mutation frequency of 2.6 or 2.7 mutations/Mb, ≈ 4.6 to 5.0 million mutations were generated in the *L. japonicus* or *M. truncatula* TILLING populations, respectively. With the goal of generating greater than 5 million mutations in common bean and assuming a mutation frequency of 2 to 3 mutations/Mb, like with *L. japonicus* and *M. truncatula*, a population of 5000 lines would generate between 5.9 and 8.9 million mutations. This number of total mutations falls between that of diploid genomes such as *A. thaliana*, *L. japonicus*, and *M. truncatula* and the more complex, polyploid cereal genomes (Table 3). The cereal genomes such as barley, maize (*Zea mays*), and wheat (*Triticum aestivum*) have a higher content of repetitive DNA, more duplication, and larger, polyploid genomes, thus requiring higher mutation density.

Over 3000 M3 lines derived from treatments using 35, 40, 45, and 50 mM EMS have been generated in genotype BAT 93. Over 2000 M3 lines have been grown in the field, generating M4 seed. DNA has been extracted and stored for TILLING from over 2000 M2 families. Phenotypic screening of 3000 M2 plants has shown a lower frequency of visible mutants in plants from the 35 mM treatment compared with those from the 40- to 50 mM treatments (Table 4). The most frequent mutations have been in leaflet shape, leaflet number, and seed dormancy, three traits that have proven difficult to fix in the M4 generation and which may have resulted from residual mutagen effects visible in the M2 generation only. More stable mutations have been observed for leaf chlorosis, seedcoat color, semi-dwarfing, and dwarfing and are generally inherited as recessive mutations. Late-maturing and spindly growth mutants have also been observed but at a lower frequency. An additional 384 M2 lines from the same population have been tested for nodulation and 33 nodulation-deficient plants were identified. All the M2s characterized as non-nodulating were found to resemble wild-type plants in the subsequent, M3, generation.

Discussion

Based on overall survival, plant development, yield of treated seed, and phenotypic mutation rate, 40 mM EMS is an appropriate concentration for the generation of a mutant population suitable for TILLING in genotype BAT 93. Using this concentration, a maximum number of mutations can be generated with a minimum population size and with relatively high efficiency. Higher concentrations of EMS resulted in overall survival rates of less than 10%, which are considered inadequate for efficient mutagenesis and population development, whereas lower concentrations (less than 35 mM) resulted in a reduction in the frequency of phenotypic mutants. Overall survival is a better criterion than germination to evaluate EMS mutagen concentration, because EMS-treated seedlings that survive germination often do not produce seed.

Table 2. Germination, plant height, yield components, and survival of common bean genotype BAT 93 after treatment with 0 to 60 mM ethyl methane sulfonate (EMS) using the Pankhurst et al. (2004) protocol.^z

EMS concn (mM)	Germination ^y 7 d (%)	Germination 14 d (%)	Plant ht 14 d (cm)	Vegetative dry wt (g/plant)	Pods (no./plant)	Seed wt (g/plant)	Harvest index	Survival of transplants ^y (%)	Survival overall (%)
0	100.0	100.0	28.4	9.1	28.7	15.6	0.63	100.0	100.0
20	83.0	92.5	18.9	17.2	19.3	6.3	0.26	96.7	89.4
30	50.6	71.2	9.8	17.0	10.7	2.9	0.13	78.0	55.6
40	19.4	49.0	6.3	13.8	8.1	1.9	0.10	67.8	33.2
50	4.2	21.1	5.2	7.9	6.0	1.6	0.07	40.7	8.6
60	2.5	7.2	10.1	4.1	4.0	1.6	0.06	20.4	1.5
Mean	43.3	56.8	13.1	11.5	12.8	5.0	0.21	67.3	48.1
LSD (0.05)	12.7	14.6	5.7	5.4	5.4	2.2	0.09	22.2	10.8

^zLeast significant difference at $\alpha = 0.05$ [LSD (0.05)] was used to compare the mean of each trait by EMS concentration treatment.

^yThe control (0 mM EMS) treatment was adjusted to 100% germination and survival, and the other treatments adjusted accordingly.

Table 3. Estimation of chemically induced^z point mutations in different plant and animal mutant populations based on published mutation frequencies.

Species	Common name	Ploidy	Mutation frequency (no./megabase)	Genome size (megabases) ^y	Estimated mutations per haploid genome (no.)	Mutant population size (no.)	Estimated mutations in population (million)	Reference ^x
<i>Hordeum vulgare</i>	Barley	2	1.0	5,439	5,439	4,600	25.0	Caldwell et al., 2004
<i>Sorghum bicolor</i>	Sorghum	2	2.0	750	1,500	1,600	2.4	Xin et al., 2008
<i>Zea mays</i>	Maize	2	2.0	2,671	5,342	10,000 (goal)	53.0	Wu et al., 2005
<i>Oryza sativa</i>	Rice	2	2–3	490	980–1,470	6,000 (goal)	5.9–8.8	Till et al., 2007; Wu et al., 2005
<i>Lotus japonicus</i>	Miyakogusa	2	2.6	466	1,212	3,800	4.6	Udvardi et al., 2005
<i>Medicago truncatula</i>	Strong-spined medick	2	2.7	466	1,236	4,032	5.0	Javot et al., 2007
<i>Caenorhabditis elegans</i>	none	2	3.4	100	3,400	1,500	5.1	Gilchrist et al., 2006
<i>Danio rerio</i>	Zebrafish	2	4.3	1,750	7,525	2,679	20.2	Draper et al., 2004
<i>Arabidopsis thaliana</i>	Mouse-ear cress	2	5.9	157	926	3,000	2.8	Greene et al., 2003
<i>Drosophila melanogaster</i>	Fruitfly	2	6.4	180	1,152	2,086	2.4	Winkler et al., 2005
<i>Triticum turgidum</i>	Durum wheat	4	25.0	12,030	300,750	768	231.0	Slade et al., 2005
<i>Triticum aestivum</i>	Bread wheat	6	40.0	16,979	679,160	1,152	782.4	Slade et al., 2005

^zEthyl methane sulfonate (EMS) used as the mutagen in all species listed except for zebrafish where *N*-ethylnitrosourea (ENU) was used.^yAnimal genome size converted from C-values to megabases by multiplying $\times 1000$. Plant genome size information from data presented by Bennett and Leitch (2005) and animal genome size data from Gregory (2008).^xMutation frequency per megabase calculated based on mutation frequencies in referenced publications.Table 4. Mutation type and observed frequencies in the common bean genotype BAT 93 mutant population of 3000 second generation (M2) plants.^z

Mutated tissue	Mutant type	Frequency (%)	
		35 mM EMS ^y	40–50 mM EMS
Leaf	Chlorotic	0.00	1.53
	Leaflet number	0.11	6.57
	Leaflet shape	0.11	13.41
Stem	Dwarf ^x	0.00	1.97
	Semi-dwarf	1.15	0.92
	Spindly	0.00	1.14
	Embryo lethal/dormant	2.99	7.62
Seed	Late maturity	0.00	5.04
Whole plant	Nodulation deficiency ^w	ND ^v	9.50
Root		0.00	0.88
Other mutations			

^zThe mutations were generated through seed treatment using ethyl methane sulfonate (EMS).^yOf the 3000 M2 plants, 844 were generated from 35 mM EMS and 2156 from 40 to 50 mM EMS treatments.^xDwarf plants were severely reduced in size and often did not produce seed, whereas semidwarf plants were intermediate in stature and generally did produce seed.^wNodulation mutant frequencies based on the 3000 M2 plants and not on the 348 lines mentioned in the text.^vND = not determined.

In comparison with mutation frequencies and effective population sizes in other species, a population of 5000 mutant lines is expected to be adequate for genome saturation and effective TILLING of *P. vulgaris* genotype BAT 93. This estimate is based on the predicted mutation frequency of two to three mutations/Mb per haploid complement for such diploid plants as *A. thaliana*, *L. japonicus*, and *M. truncatula* as well as that of the more complex, polyploid cereal genomes. Using concentrations of between 35 and 50 mM EMS, over 3000 M3 lines have been developed that show morphological and phenological mutations at reasonable frequencies. Although mutation frequencies were estimated using phenotypic traits, the actual mutation frequency for BAT 93 will be calculated based on TILLING experiments at specific loci through comparison with natural rates of polymorphism in bean (e.g., Slade et al., 2005; Till et al., 2007). Functional redundancy in specific species such as *A. thaliana* could confer greater tolerance to high mutation load as has been found with yeast (*Saccharomyces cerevisia*) (Gu et al., 2003). If common bean is similar to other small genome legumes such as *M. truncatula* and *L. japonicus* with relatively low functional redundancy, it is expected to be less tolerant to high mutation load.

EMS mutagenesis has been shown to be effective in common bean, through isolation of a low phytic acid mutant in another EMS mutant population (Sparvoli et al., 2007). In addition to reverse genetic applications, forward genetic screening of mutants has been used to isolate specific mutants using the BAT 93 mutant population [e.g., for those perturbed in embryogenesis (Silue et al., 2006)]. If identified, beneficial mutations from the population could be directly incorporated into breeding programs using the same molecular tools such as DNA sequence and polymerase chain reaction primers developed as part of the TILLING protocol. Furthermore, because EMS-generated mutant lines are not transgenic, novel lines with agronomically beneficial traits could be incorporated into

breeding programs without additional hurdles. Once the BAT 93 mutagenesis population developed from this research is completed and the TILLING protocol is optimized for common bean, the population will be used for genetic analysis and will be made available to the research community for the evaluation of specific loci and traits of interest.

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