Agricultural Sciences Journal
Available online at http://asj.mnsuam.edu.pk/index.php
ISSN 2707-9716 Print
ISSN 2707-9724 Online
https://doi.org/10.56520/asj.v8i1.585

Research Article

OPTIMIZATION OF IN-PLANTA TRANSFORMATION EFFICIENCY IN MUNGBEAN (VIGNA RADIATA L.) USING AN AGROBACTERIUM-MEDIATED APPROACH

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Abstract

In *planta* transformation is a promising technique for genetic modification in mungbean *(Vigna radiate* L.), yet its efficiency is influenced by various factors. This study aimed to assess the impact of genotype, *Agrobacterium* culture concentration, and floral bud stage on transformation efficiency. The transformation process was evaluated using β-glucuronidase (GUS) staining, wherein successfully transformed embryos exhibited a blue coloration. The expression of the GUS gene was confirmed through visual examination and microscopic analysis. Results indicated that genotype significantly influenced transformation efficiency, with NM-19 exhibiting the highest mean transformation rate (6.0758%), whereas NCM-2013 had the lowest (0.050%). Similarly, *Agrobacterium* culture concentration played a crucial role, where an optical density (OD) of 1.0 at the late floral bud stage yielded the highest transformation efficiency (5.3483%). Conversely, lower transformation rates were observed at an OD of 0.5 during the early floral bud stage (2.9258%). The interaction between genotype and culture concentration was also significant, with NM-19 at OD 1.0 achieving the highest transformation efficiency (6.5500%). Additionally, the late floral bud stage resulted in significantly higher transformation efficiency compared to the early floral bud stage. Statistical analysis using ANOVA confirmed the significant effects of genotype, culture concentration, and floral bud stage on transformation efficiency. The findings suggest that selecting an appropriate genotype, optimizing bacterial culture concentration, and targeting the late floral bud stage can enhance transformation success in mungbean. These insights contribute to improving genetic transformation protocols for mungbean and may facilitate advancements in legume biotechnology.

Keywords: *Mungbean, Agrobacterium, Microscopic analysis, Genotype*.

(Received: 03-Feb-2026 Accepted: 18-May-2026) Cite as: Rasheed. S., Ul. H. Mahmood., S. L. Muhammad., M. Iqra., B. Abdul. 2026. Optimization of in-planta transformation efficiency in mungbean (*vigna radiata* L.) Using an agrobacterium-mediated approach. Agric. Sci. J. 8(1): 21-31.

1. INTRODUCTION

Mungbean (*Vigna radiata* L.) is a leguminous crop that is grown extensively in the subtropical and tropical region for its high protein seed and sprout (Diatta *et al.*, 2024). It is highly appreciated for its high levels of pro-vitamin A and phosphorus, low production of flatulence and ease of digestibility of its seeds (Manu *et al.*, 2023). Moreover, mungbean has comparatively low amount of anti-nutritional factors and a favourable amino acid profile with high lysine and low methionine content which complements the low lysine and high methionine content of cereals to make a balanced diet (Balaso, 2024). Mungbean is an agronomically beneficial short-duration crop that can fix nitrogen and is an effective

component in crop rotations to enhance soil nitrogen levels and the break of pest and disease cycles (Singh *et al.,* 2025). Though, there is a continuous breeding program, mungbean production has not increased because of its high vulnerability to different abiotic and biotic factors. Salinity, drought, fungal, bacterial and viral pathogens and insect pests are factors that limit its yield (Duan *et al.*, 2025). Also, morphological and physiological limitations including indeterminate growth, asynchronous flowering, sensitivity to photoperiod and temperature, low harvest index, low germination rates, and pod shattering reduce productivity. The application of agrochemical products can improve the quality of the crop but if excessive amounts are used it poses environmental concerns. The small size of the haploid genome (0.48–0.53 pg) of mungbean makes it a suitable plant for biotechnological and physiological research. Although mungbean production of the Indian subcontinent accounts for more than 66% of the total world production, the yield has not been improved significantly during the last 30 years because of its high susceptibility to biotic and abiotic stresses (Samanta *et al.,* 2025). Research has been conducted on breeding and genetic engineering approaches to improve the agronomic characteristics of mungbean (Kanmani Bharathi *et al.*, 2026). Traditional breeding methods have been less successful however, because desirable genes are not available in closely related species, genetic transformation offers a more promising approach to enhancing resilience and productivity (Loizou *et al.*, 2025). For all the transformation methods available, the genetic transformation using *Agrobacterium* has been widely used because of its efficient stable DNA integration (Abdullah *et al.*, 2025). In the last 30 years, however, many advances have been made in the genetic transformation of grain legumes (Sarroukh *et al.,* 2025). Despite this, an optimized screening system for transgenic legumes must be established to guarantee the stability of the transgene, fertility and low somaclonal variation (Belaffif *et al.,* 2025; Gélinas Bélanger, 2025). In view of the difficulties, there is an alternative approach available, namely, in planta transformation which circumvents in vitro culture and regeneration, and may be more efficient for transformation in *Vigna radiate* (Vacu *et al.,* 2025). This study introduces a protocol for genetic transformation of *Vigna radiata* using *Agrobacterium tumefaciens* which overcomes major drawbacks of genetic transformation in large-seeded legumes.

2. MATERIALS AND METHODS

2.1. Agrobacterium Strains, Binary Vectors, and Plant Materials for Genetic Transformation

Eight mungbean varieties were developed in Pulses Program, National Agriculture Research Centre (NARC), Islamabad. The plants were grown in pots in a completely randomized design with three replications of these genotypes. *Agrobacterium* strain LBA 4404 with the pCAMBIA1301 vector, was utilized for the transformation by floral injection method (Alam, 2026). The plasmid carried the gene for GUS which was driven by the CaMV 35S promoter (Kumar *et al.*, 2026).

2.2. Bacterial Culture and Floral Injection-Based Transformation

Agrobacterium was grown for overnight in YEP medium (pH 7.2) supplemented with 50 mg/mL Kanamycin and 10 mg/mL Rifampicin (Mohana *et al.*, 2025) in a 210 rpm incubator shaker at 28°C. The culture was centrifuged for 10 minutes in an ultracentrifuge at 10,000 rpm to collect the pellet. The antibiotic-free MSO medium was used to resuspend the pellet. Adjusted half of the culture to OD 1.0 and the other half to OD 0.5 by using spectrophotometer. The method was used with some modifications was used

for in planta transformation (Akbulut, 2025). The culture was introduced into the floral buds at two stages; stage 1 (early floral bud) and stage 2 (late floral bud). Treated buds were tagged to denote the date and floral bud stage (Tian *et al.*, 2025) for identification.

2.3. Factors affecting in planta transformation

The effect of genotype, floral bud stage and culture concentration on the factors affecting transformation were studied. Ten genotypes (NM-19, Chakwal-06, 13Tm-04, Ncm-2013, 07008, NM-2011, 09Tm-11, and 13Tm-14) were used for transformation. The floral buds were injected at two stages: Stage 1 (small green) and Stage 2 (larger pale). Further, 0.5 OD and 1.0 OD at 600 nm absorption were also used for the floral injection.

2.4. Gene expression

The plants were harvested at maturity and the transformation rate was evaluated by a GUS assay method (Yuan *et al.*, 2025). The GUS buffer solution (Solution A) was prepared by dissolving 372 mg EDTA and 780 mg NaH_2PO_4 in 80 mL of distilled autoclaved water, followed by the addition of 10 µL Triton X-100, adjusting the volume to 100 mL with distilled autoclaved water, and setting the pH to 7.0. The X-Gluc solution (Solution B) was prepared by dissolving 10 mg X-Gluc in 100 µL DMSO in an Eppendorf tube. For the reaction mixture, 1 mL of GUS buffer was mixed with 5 µL X-Gluc solution at the time of use. Autoclaved water and bottles were used for both solutions which were stored separately at 4°C until use. The GUS assay included sowing of transformed seeds in pots, cutting the germinated shoots into small pieces and overnight incubation at 37°C in the GUS staining solution which was removed after 12 hours and stored at 4°C in a 1:1 glycerol-ethanol solution.

2.5. Statistical analysis

The experiment was laid out in a factorial completely randomized design (CRD) with the data analyzed using Analysis of Variance (ANOVA) and Least Significant Difference (LSD) test by using the software STATISTIX 8.1 (Steel *et al.*, 1997).

3. RESULTS

The transformed genotypes were tested by using a GUS staining solution and the transformed embryos were blue color immersion in the solution. The two GUS solutions were combined and shoot tips were dipped in it and incubated for overnight at 37°C, and then gene expression was confirmed by visual examination or microscopic observation.

3.1. Factor affecting transformation

Several factors affect the transformation efficiency, such as *Agrobacterium* strains, plant genotype, culture concentration and the stage of floral bud and the effect of these factors on the transformation efficiency is discussed and analyzed.

3.2. Effect of genotypes

Table 1 showed that the genotypes exhibited highly significant effect on the transformation rate. The comparison result of LSD showed that NM-19 (genotype no. 1) gave the highest mean value (6.0758) followed by NM-18 (genotype no. 2) with a mean value of (4.2873) and NCM-2013 (genotype no. 4) with a mean value of (0.500). The results suggest that NM-19 has the highest transformation efficiency and is the most convenient genotype for achieving the highest transformation efficiencies among those tested. Our findings are consistent with previous reports on the effect of genotype on transformation rates in Italian ryegrass (Lee *et al.*, 2010), and on the effect of genotype on transformation efficiency (Kahrizi *et al.*, 2006).

3.3. Effect of culture concentration

The results of the analysis of variance indicated that culture concentration had a highly significant effect on transformation efficiency, as presented in Table 2. The mean values, calculated based on the number of

floral buds injected per treatment, were compared using the LSD test (Table 2. Among the treatments, Treatment 1 (OD: 1.0; late floral bud stage) exhibited the highest transformation efficiency (5.3483), followed by Treatment 4 (OD: 1.0; late floral bud stage). In contrast, the lowest mean value (2.9258) was observed in Treatment 4 (OD: 0.5; early floral bud stage).

Studies on *Arabidopsis* have shown that bacterial concentration does not significantly impact transformation efficiency; whereas transformation in *Nicotiana tabacum* was significantly influenced by bacterial concentration these findings suggest that the effect of *Agrobacterium* concentration on transformation efficiency is species-specific. Setiaet *et al*., reported that a culture concentration of OD 0.6 significantly affected transformation efficiency in pigeon pea. Similarly, a culture concentration of OD 0.8 had a significant impact on transformation efficiency in oil palm. Notably, OD 0.8 also yielded significant results in winter jujube, sugarcane, and *Acacia crassicarpa* (Setia *et al.*, 2003).

3.4. Effect of interaction of genotype and culture concentration

The results of comparing mean values by using the LSD test showed that genotype Nm-19 had the greatest mean value (6.5500) for transformation at a culture concentration of 1.0 OD. In contrast, Ncm-2013 recorded the lowest mean values at both 0.5 OD (0.0167) and 1.0 OD (0.0833). The results show that genotype x culture concentration effects are significant in determining transformation efficiency.

Table 1: Mean Comparisons of Different Genotypes for Transformation by Using LSD Test

No.	Genotype	Means (%)	Homogeneous Groups
1	NM-19	6.0758	A
3	13Tm-04	5.2750	B
7	09Tm-11	5.0542	C
5	07008	5.0075	C
8	13Tm-14	4.7825	D
2	Chakwal-06	4.6067	E
6	NM-2011	4.4733	F
4	Ncm-2013	0.0500	G

Table 2: Mean Comparisons of interactions (Culture conc. X bud stage) for Transformation by Using LSD Test

No.	Treatment	Means (%)	Homogeneous Groups
1	Late floral bud stage Culture conc.1.0OD	5.9321	A
2	Late floral bud stage Culture conc.0.5OD	4.7646	B
3	Early floral bud stage Culture conc.1.0OD	4.0400	C
4	Early floral bud stage Culture conc.0.5OD	2.9258	D

Table 3: Mean Comparisons of interactions (floral bud stage) for Transformation by Using LSD Test

Floral bud stage	Mean	Homogeneous Groups
Late floral bud stage	5.3483	A
Early floral bud stage	3.4829	B

Table 4: Mean comparisons of interactions of transform for genotype, culture concentration by using LSD test

Genotype	Culture concentration	Mean	Homogeneous Groups
Nm-19	2	6.5500	A
13Tm-14	2	6.0317	B
13Tm-04	2	6.0167	B
09TM-11	2	5.6183	C
NM-19	1	5.6017	CD
07008	2	5.4983	D
Chakwal-06	2	5.1017	E
Nm-2011	2	4.9883	E
13Tm-04	1	4.5333	F
07008	1	4.5167	F
09Tm-11	1	4.4900	F
Chakwal-06	1	4.1117	G
Nm-2011	1	3.9583	H
13Tm-14	1	3.5333	I
Ncm-2013	2	0.0833	J
Ncm-2013	1	0.0167	J

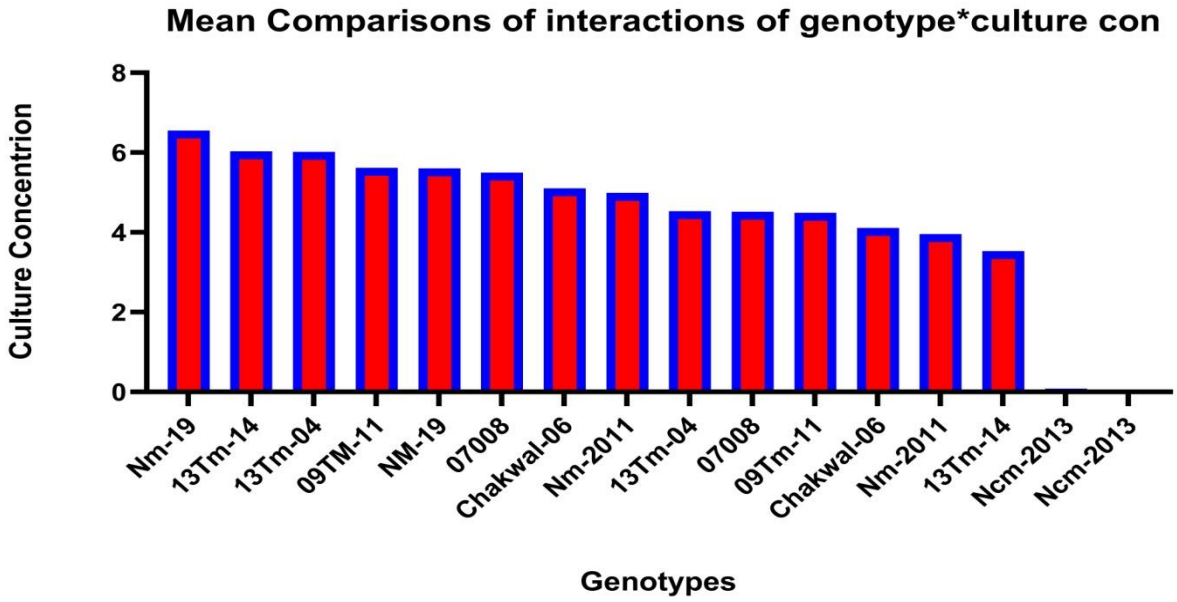

Figure. 1: Interaction effect of genotype and *Agrobacterium* culture concentration on transformation efficiency (%) in mungbean (*Vigna radiata* L.).

3.5. Effect of interaction of genotype, culture concentration and floral bud stages

Influence of the combination of genotype, culture concentration, and flower bud stage plays an important role in determining the extent of the observed effect, with Nm-19 (2, 2) being the highest (8.0000), while Nm-19 (1, 2) and 13Tm-04 (2, 2) follow. Genotypes like Ncm-2013 had minimal effect under all

test conditions, which implies a clear genotype-specific influence. It appears that culture concentration and flower bud stage play an equally important role in influencing the genotype's effect in which genotypes have been found to produce higher effect when both factors were set at 2.

Table 5: Mean comparison of interaction of transformation for genotype *culture concentration. *floral bud stage by using LSD Test

Genotype	Culture concentration	Floral bud stage	Mean	Homogeneous groups
Nm-19	2	2	8.0000	A
Nm-19	1	2	7.1033	B
13Tm-04	2	2	7.0000	B
07008	2	2	6.9967	B
13Tm-14	2	2	6.9967	B
09Tm-11	2	1	6.2367	C
Chakwal-06	2	2	6.1033	C
07008	1	1	6.0000	C
Nm-2011	2	1	5.9567	C
Nm-19	2	2	5.1000	D
Chakwal-06	1	1	5.0833	D
13Tm-14	2	2	5.0667	D
13Tm-04	2	2	5.0333	D
13Tm-04	1	2	5.0000	D
09Tm-11	2	1	5.0000	D
13Tm-14	1	1	5.0000	D
09Tm-11	1	1	4.9800	D
Nm-2011	1	1	4.9167	D
Nm-19	1	1	4.1000	E
Chakwal-06	2	1	4.1000	E
13Tm-04	1	1	4.0667	E
Nm-2011	2	1	4.0200	E
07008	2	1	4.0000	E
09Tm-11	1	1	4.0000	E
Chakwal-06	1	1	3.1400	F
07008	1	1	3.0333	F
Nm-2011	1	1	3.0000	F
13Tm-14	1	1	2.0667	G
Ncm-2013	2	2	0.1667	H
Ncm-2013	1	2	0.0333	H
Ncm-2013	1	1	0.0000	H
Ncm-2013	2	1	0.0000	H

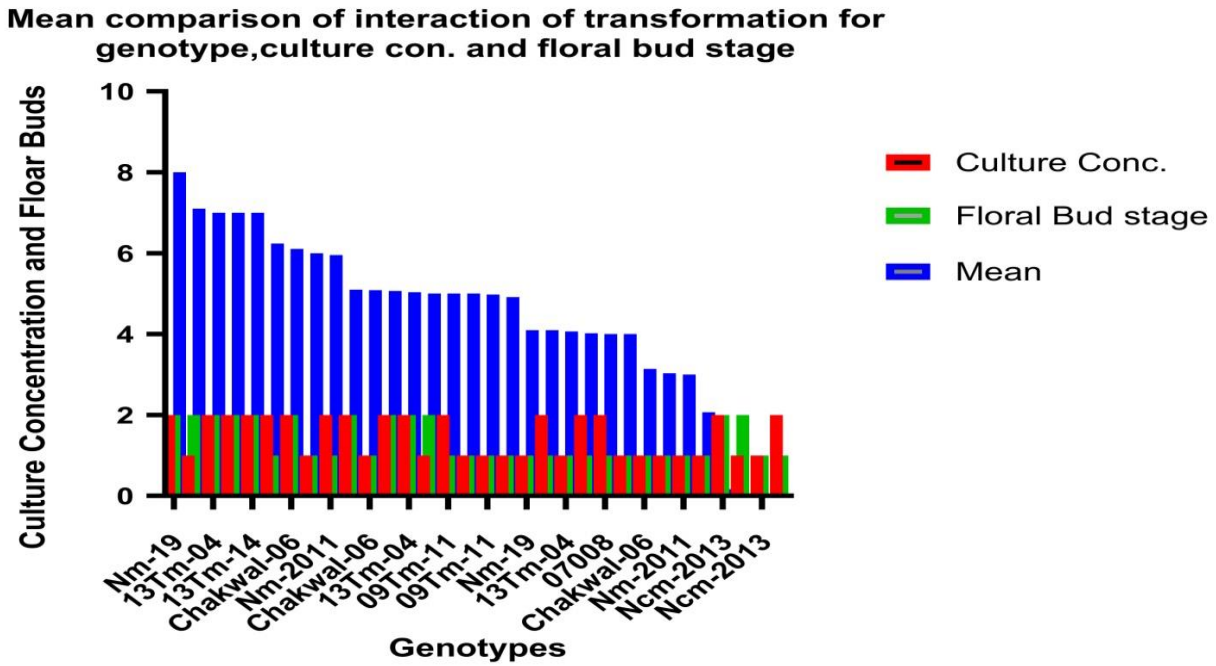

Figure. 2: Interaction effect of genotype, *Agrobacterium* culture concentration, and floral bud stage on transformation efficiency (%) in mungbean (*Vigna radiata* L.).

4. DISCUSSION

The present study has shown that genotype, Agrobacterium culture concentration and the stage of floral bud have significant effect on transformation efficiency in mung bean plant. Of the eight genotypes investigated, the best response was obtained in NM-19 and the poorest response in NCM-2013. These results suggest that genetic background is an important factor in determining infection susceptibility to Agrobacterium and subsequent integration of T-DNA. In mungbean, Jaiwal *et al*., (2001) and Mahalakshmi *et al*., (2006) reported genotype dependent transformation efficiency; some genotypes being more competent for Agrobacterium mediated gene transfer. Similarly, Lee *et al*., (2010) found that there were significant differences in transformation among Italian ryegrass genotypes, indicating the need to choose the appropriate host genotype for successful transformation. The superior performance of NM-19 observed in present study indicated that this genotype had favorable physiological and genetic characteristics that would help them to be efficient gene transfer and expression. The transformation efficiency was also significantly affected by the concentration of the agrobacterium culture. The higher the bacterial density (1.0 OD), higher the transformation as compared to 0.5 OD. This could be explained by the enhanced availability of bacterial cells to attach and transfer of the T-DNA to the plant tissues. Optimized levels of bacteria have been seen to improve the transformation frequency in rape (Kahrizi *et al*., 2007) and pigeon pea (Basavanna 2003). Jaiwal *et al*., (2001) also reported an optimum level of Agrobacterium to be used for transformation to improve the efficiency of transformation in mungbean. But, slightly high count of bacteria may result in tissue necrosis and bacterial overgrowth, leading to a decrease in transformation efficiency (Joyce *et al*.,

2010). Thus, the improved transformation rate detected at 1.0 OD in the current study reiterates the need for optimal inoculum density in order to achieve efficient gene delivery. The developmental stage of the floral buds was an important factor to consider: Late stage floral buds had higher transformation efficiency as compared to early stage floral buds. This result suggests that the reproductive tissues in advanced developmental stages are more susceptible to Agrobacterium infection and T-DNA integration. Martinez-Trujillo *et al*., (2004) showed that developmental stage of the flowers is a crucial factor in determining the transformation success in Arabidopsis. Desfeux *et al*., (2000) have also proposed that developing ovules are the major targets for A. tumefaciens-mediated transformation in the floral mediated transformation system. This may be related to the increased ease of access to reproductive tissues and increased cellular competence for foreign DNA uptake with the use of late floral buds in the present study. Another significant interaction between genotype and culture concentration and flower bud stage was seen. The genotype NM-19 that showed maximum transformation efficiency (8.0%) had a late floral bud injection with Agrobacterium culture at 1.0 OD. The results presented here show that individual factors are not enough for successful transformation; combined effects are also important. Kahrizi *et al*., (2007) reported similar results in rapeseed and Gu *et al*., (2008) in winter jujube, where the transformation efficiency was significantly influenced by the interaction between genotype and conditions of bacteria inoculum. The present results thus highlight the importance of creating an optimized host genotype, bacterial cell number, and developmental stage of the target tissue for the most efficient transformation. The expression of the GUS reporter gene in transformed tissues was successful, and confirmed that foreign DNA was transferred and expressed in tissues. The blue coloration of the transformed shoots which did not appear in the negative control confirmed the successful transformation by Agrobacterium. In mungbean, similar GUS expression patterns have been reported by Jaiwal *et al*., (2001) and Mahalakshmi *et al*., (2006) who had employed histochemical GUS assays as reliable indicators of successful transformation events. The positive GUS expression obtained in the present study clearly indicates that floral injection is a simple, reliable and effective method for in planta transformation of mungbean and can be used in future genetic improvement program for developing cultivars resistant to diseases, insects and stresses.

5. CONCLUSION

Mungbean is a nutritious leguminous plant widely used in agriculture. However, the productivity of this valuable crop can be greatly affected by many factors, both biological and environmental. Using traditional methods in plant breeding for resistance to pathogens and other negative impacts may take much time. Genetic engineering appears as the promising tool for further enhancement of plants' qualities. Nonetheless, the problem associated with difficult shoot regeneration in vitro makes mungbean transformation rather complicated. This research has shown us how to optimize in planta transformation techniques for insertion of GUS gene into the genome of this leguminous crop. It has been found that the use of floral bud injection in mungbean through a micropipette works better than other methods. The genotype NM-19 turned out to have the highest transformation rate among all genotypes used. Also, a combination of the late floral bud stage with the culture of 1.0 OD has resulted in a more efficient transformation. The data generated here is crucial in helping understand the best approaches that can be

taken towards achieving optimization of in planta transformation methods for mungbean genetic enhancement. More studies need to focus on making further improvements towards achieving higher efficiency in transformation and determining the stable expression of transgenic material. The findings in this study form an important foundation toward creating genetically enhanced mungbeans.

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