Genetic variations of EMS-induced chili peppers (*Capsicum annuum*) cv. Gelora generate geminivirus resistant mutant lines

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Abstract.A mutation breeding program was conducted to improve chili pepper (*Capsicum annuum*) resistance to Geminivirus caused by *Pepper yellow leaf curl virus* (PepYLCV). The disease can cause significant yield losses on chili pepper. This study was conducted to enhance genetic variation in pepper cv. Gelora using ethyl methanesulphonate (EMS) to obtain mutant lines resistant to PepYLCV. Exposure to an EMS solution of 0.5% (v/v) for 30 minutes was used for mutagenizing shoot tips to produce the first population (M_1). After the treatment, shoot tips were cultured on MS medium to regenerate planlets that were subsequently acclimatized to produce M_2 population. A set of 2,955 mutant lines (M_2) were screened in a greenhouse by inoculating PepYLCV isolate Segunung using white flies (*Bemisia tabaci*) as the virus vector. Six weeks after inoculation, the M_2 population was examined for symptoms of Geminivirus and was verified for the presence of PepYLCV infection by PCR using specific primer. The greenhouse assay successfully selected 47 lines (1.6%) of M_2 mutant population that showed no symptoms and no infection to PepYLCV compared to Gelora and Tanjung-2 varieties. Subsequent field test of M_3 mutant lines in Geminivirus endemic area in Lembang, West Bandung, revealed that 15 out of 47 lines showed high resistance to PepYLCV and have good agronomic characters including yield components. A set of primers corresponding to the PepYLCV infection which produced 678 bp in size indicated its good amplification in the susceptible mutant lines, confirming the PepYLCV resistance of 15 selected mutant lines. The resistant mutants were immune to PepYLCV and had potential to be released as improved varieties in the future.

Keywords: *Capsicum annuum*, ethyl methanesulfonate, geminivirus, *Pepper yellow leaf curl virus.*

1. Introduction

Pepper yellow leaf curl virus (PepYLCV) is a pepper-devastating viral pathogen transmitted by whitefly *Bemisia tabaci* [1]. The virus belonging to the family Geminiviridae and genera of Begomovirus have become a serious constraint to chili pepper in tropical and subtropical areas worldwide [2,3]. Chili plants infected with PepYLCV show severe symptoms such as stunting with small, thick, curly leaves and yellowing along leaf margins, which lead to significant fruit loss ranging

from 30 to 100% [4]. First detected on tobacco plants in the East Java in 1932 [5], PepYLCV is now endemic in many provinces of Indonesia and most commonly found in chili pepper and several horticultural crops. Although there is no national data of yield losses of chili caused by Geminivirus infection, Direktorat Perlindungan Tanaman Hortikultura [6] reported that the economic loss of chili due to PepYLCV reached IDR 20 billion in 14 provinces in 2007. In 2009, PepYLCV affected 650 ha of chili plants and caused yield loss up to IDR 16 billion in Kediri, East Java [6]. This disease can spread quickly in correlation with the increase population of *B. tabaci*.

So far, various methods have been used to control PepYLCV distribution, i.e. improvement of cultivation techniques, eradication of infected plants, prevention of vector dispersal and management of vector population using pesticides [4]. However, there are no effective methods to control this disease and its insect vectors. A single whitefly insect can transmit the virus to the plants. Therefore, the use of PepYLCV-resistant plant variety is the most promising method to control this disease. Host resistance plant is mostly preferred besides it is an effective, economical and environmentally friendly method for disease control [7]. Conventional breeding of PepYLCV-resistant plant was conducted through the selection of germplasms to identify possible sources of resistance genes to PepYLCV. As non-center of chili origin, Indonesia has low genetic variability of chili peppers, and it is a great challenge for breeders to collect or to develop a large number of germplasm resources.

Mutations are the primary source of all genetic variations existing in any organism, including plants [8]. Mutation breeding involves the development of new varieties by generating and utilizing genetic variability through chemical, physical and biological mutagenesis [9]. Chemical mutagens are preferably used to induce point mutations [10] and to generate not only loss-of-function, but also gain-of-function phenotypes if the mutation leads to a modified protein activity or affinity [9]. In addition, chemically-induced mutation showed high efficiency in producing individual lines that bear single point missense and non-sense substitutions in hundreds of genes [11]. Among the chemical mutagens, ethyl methanesulfonate (EMS) is considered as an effective one because it can form adducts with nucleotides efficiently, resulting in mispairing among these nucleotides with their complementary bases, and thus, introducing base changes after replication [12].

Previous studies found that EMS is an effective mutagen and can be used to improve desired identifiable characters such as resistance to virus diseases. *Arabidopsis* mutant lines bearing EMS-induced null alleles in the eukaryote initiation factor eIF(iso)4E gene are resistant to infection by several viruses from the genus Potyvirus, including *Turnip mosaic virus* (TuMV), *Lettuce mosaic virus* (LMV) and *Tobacco etch virus* (TEV) [13,14]. Mutagenesis using EMS also caused loss-of-susceptibility due to point mutation of eIF4E gene in *Capsicum annuum* to TMV [15], while in rice caused loss of susceptibility to TMGMV and PMMoV, and *Solanum lycopersicum* to AltMV [16]. EMS-induced mutations in tomato eIF4E were identified by sequencing eIF4E genes from 3,008 M_2 population [17] and by the TILLING of 4,759 M_3 population [18]. Loss of interaction of virus with eIF4E is correlated with a loss of infectivity of the virus, suggesting that the interaction is critical for virus production [19,20].

Induced mutations can rapidly create variability in quantitatively and qualitatively inherited traits in crops. Therefore, the objective of this study was to obtain new germplasm resistant to PepYLCV by the improvement of Gelora chili pepper cultivar.

2. Materials and methods

2.1. EMS mutagenesis

This experiment used Gelora, a red pepper cultivar (*C. annuum* L.) with medium yielding ability but susceptible to PepYLCV. Certified seeds were obtained from PT Sinar Bumi, East Java. The seeds were germinated in 72-hole seedling trays. One hundred shoot tips from 21-day-old seedlings were pre-soaked in disinfectant solution for 1 minute. Shoot tips were treated for 30 seconds at room temperature on a shaker with 0.5% (V/V) of EMS prepared in a 0.1 M phosphate buffer at pH 7.0. Shoot tips were then thoroughly washed three times with sterile distilled water and transferred to vessels containing MS medium supplemented with 2.4-D 3 mg/l and Thidiazuron 0.5 mg/l. Vessels

were kept in a growth chamber at 25±2°C under white light until growing up. Rooting induction was conducted in vessels containing 60 ml of solid MS½N supplemented with NAA 0.5–1.0 mg/l. Subsequently, rooted-plantlets were acclimatized in a screen house at a temperature ranging from 25°C to 30°C. Plantlets of 6 to 8 cm in length were transferred to seed pots (9 cm^2 area) containing a sand and humus mixture (2:1). To maintain humidity, the plantlets were covered with plastic caps and gradually opened during two-week acclimatization. Survival of plants was monitored daily and maintained with recommended cultural practices until fruit harvested. First mutant lines (M_1) seeds were harvested and planted to generate M_2 population.

2.2. Screening for putative resistant mutant lines

A total of 2,955 M_2 of mutant lines were screened to PepYLCV under greenhouse condition at Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development. Gelora, Chiko and Tanjung-2 were used as control varieties. The seeds were germinated in 72-hole seedling trays. The plants were maintained in cages (250 cm × 110 cm × 100 cm) covered with 100-micron mesh cloth. Thirty-day-old plants were inoculated for a period of 48 hours with one or two *B. tabaci* insects which have acquired Begomovirus isolate Segunung for 24 hours. One week after inoculation, plants were transplanted to 9 cm × 15 cm polybags filled with soil and organic matter mixture (12:1).

Disease and agronomic parameters observed were incubation period, symptom types, disease intensity, and also the number and weight of fruits. The disease symptoms were observed during the first harvest period. The disease intensity was scored from 0 to 5 and was used to determine severity level of Begomovirus infection using the formula: DI = Σ(ni × zi)/(N × Z) × 100%, where i = 0–5 score, ni = sum of plant symptoms with score value, zi = value of symptoms score, N = sum of plant, and Z = the highest score of symptoms.

2.3. Establishment of pepper mutant lines in field

The experiment was carried out at Indonesian Vegetables Research Institute, Lembang, West Bandung, under natural infection of PepYLCV. The climate in Lembang is tropical wet and dry, with the average annual temperatures ranges from 17 to 27°C. The rainy season runs from June through October with the average annual rainfall is 1,036.9 mm. A total of 40 mutant lines was arranged in a randomized block design with three replicates. Thirty-day-old seedlings of all mutant lines were individually transplanted in plots covered with polyethylene mulch. The plot size was 1 m × 6 m with 50 cm × 70 cm planting distance. Each plot was comprised of 30 plants per plot. Plants were fertilized with 150 N, P and K with an application rate of 250, 200 and 150 kg/ha, respectively, supplied by a dripping irrigation system. No insecticide was applied to increase successful infestation of *B. tabaci*. Plants were observed for disease intensity, yield components and agronomic traits.

Data were analyzed by means of Statistical Analysis Software (SAS) programme and means were separated by the Least Significant Differences (LSD) test. Genetic variability was calculated based on previous formulas [21] (Table 1).

Table 1. Calculation method of genetic variability.

Source of variant	Degree of freedom	Mean of square	Probability[a]
Replication	[replication(r)-1]	Mean (M3)	$\sigma_e^2 + 21\sigma_u^2$
Mutant lines	[Number of mutant (m)-1]	Mean (M2)	$\sigma_e^2 + 3\sigma_g^2$
Error	(r-1) (m-1)	Mean (M1)	σ_e^2

[a] $\sigma_e^2 = enviroment$; σ_g^2 = genetic variability

$\sigma_g^2 = \frac{M2-M1}{r}$ $\sigma_e^2 = M1$; $\sigma_p^2 = \sigma_g^2 + \sigma_e^2$.

Genotypic coefficients variances (GCV) and phenotypic coefficients variances (PCV) were calculated using the formula:

$$GCV = \frac{\sqrt{\sigma_g^2}}{mean} \times 100\%, \text{ and } PCV = \frac{\sqrt{\sigma_p^2}}{mean} \times 100\%$$

The criteria for GCV and PCV relative is low, medium, and high, if x <10%, 10%< x <20%, and 20%< x, respectively[22].Heritability was calculated using the formula:

$$h_{bs}^2 = \frac{\sigma_g^2}{\sigma_g^2 + \sigma_e^2}$$

Genetic advance was determined as described by Johnson et al. [23]:

$$GA = K(\sigma_p)h^2,$$

where: K= the selection differential (K-2,06 at 5% selection intensity), σ_p = the phenotypic standard deviation of the characters, and h^2 = broad-sense heritability. The genetic advance as percentage of the mean (GAM) was calculated as described by Johnson et al. [23] as follows:

$$GAM = \frac{GA}{mean} \times 100\%,$$

where: GAM = genetic advance as percentage of the mean, and GA = genetic advance.

2.4. Virus detection

The presence of PepYLCV in the samples was detected by PCR analysis using the specific primers for PepLCV (primer PAL1v 1978: 5'-GCATCTGCAGGCCCACATYGTCTTYCCNGT-3' and primer PAR1c 715 5'-GATTTCTGCAGTTDATRTTYTCRTCCATCCA-3') to amplify a 1,600 bp fragment from the intergenic region of PepYLCV component [24]. The procedure of Dellaporta et al. [25] was used for DNA extraction. PCR reactions were performed in a total volume of 50 µl containing 2.5 mM of each dNTP, 1 µl of oligonucleotides (50 ng/µl), 1 µl of *Taq* polymerase (5 U/µl), and 250 ng plant DNA. The PCR conditions were 94°C for 2 min, 50°C for 2 min and 72°C for 2 min, for 35 cycles. The PCR products were analyzed on 0.8% agarose gels.

3. Results and discussion

3.1. Greenhouse screening

A total of 2,955 chili pepper mutant lines were screened for PepYLCV resistance to identify resistant mutant. Results of the greenhouse assay are presented in Figure 1. The data revealed that 82% of 2,955 mutant lines were infected by Begomovirus at three weeks after inoculation (WAI). Until the generative phase, there are only 5% of mutant lines showing no symptom of PepYLCV. Finally, 47 (1.6%) of pepper mutant lines were identified as resistant to PepYLCV because they showed no visual symptoms and contained no particles viruses based on PCR detection. On the contrary, susceptible lines showed systemic symptoms of PepYLCV including stunting with small, thick, curly leaves and yellowing along the leaf margins.

Such resistance responses in different mutant crops against viruses were reported in *C. annuum* mutants to *Tobacco mosaic virus* (TMV) [15], *S. lycopersicum* mutants to TMV [26], rice mutants to *Rice dwarf virus* [27], and *A. thaliana* mutants to TMV [15], *Cucumber mosaic virus* (CMV) [28,29], and *Turnip mosaic virus* [30]. In *A. thaliana*, the screening of large mutagenized populations has led to the identification of a number of mutants in which virus susceptibility is reduced or eliminated. The effect of EMS to *A. thaliana* is inducing mutation on *TOM1*, *TOM2* and *TOM3* genes that are required for efficient TMV [28] and CMV [31] replication in protoplasts. EMS induces C-to-T changes resulting in C/G to T/A substitutions, whereas methyl methanesulfonate produces T/A to G/C transversion and A/T to G/C transitions (1,3,4) [12]. Based on codon usage in *Arabidopsis*, the frequency of EMS-induced stop codon and missense mutations has been calculated to be ~5% and ~65%, respectively [12]. EMS mutagenesis generates randomly distributed mutations throughout the genome [32].

3.2. Field experiment

3.2.1. Selection of chili mutant for resistance lines. Forty-seven selected-chili pepper mutant lines were subsequently tested under natural infestation of whiteflies during the dry season in Lembang, West Bandung, West Java. The disease incidence during the experiment was quite high and evenly spread throughout the crops. Infection symptom of Geminivirus was already visible at three weeks after transplanting, and the disease incidence continued to increase. At 15 weeks after transplanting, the disease incidence on the tested lines was 65.33%, whereas on Gelora, Tanjung-2 and Chiko was 34.67, 42.67 and 32.00%, respectively. These result indicated that the experiment under natural infection of the virus in Lembang was suitable for the identification of chili pepper mutant resistant to PepYLCV. Out of 47 chili mutant lines, 15 mutants were highly resistant to Geminivirus based on visual symptoms and detection by PCR (Table 2). The resistant mutants exhibited 0–5,33% of PepYCLV infection with negative PCR detection. This study showed that mutation breeding is an important aspect in disease management practice. The resulted resistant mutant lines can be further used for selection of suitable genotypes in PepYLCV resistance breeding programme in chili.

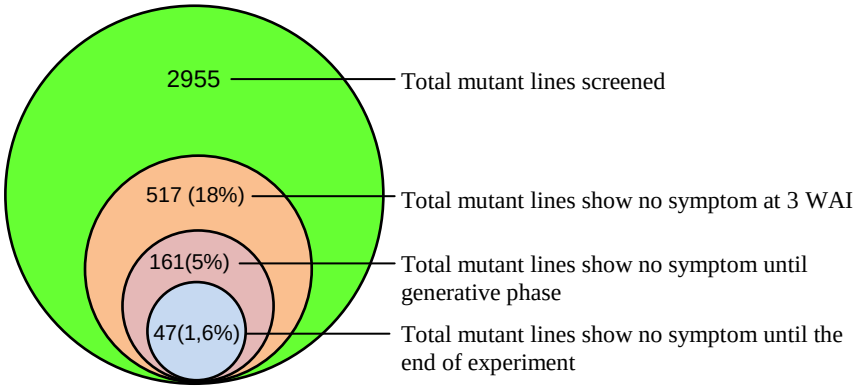

Figure 1. Results of screening of 2,955 chili pepper mutant lines against PepYLCV under greenhouse condition.

3.2.2. Analysis of variance of agronomic and yield component of chili mutant lines. Beside genetic variation in resistance against PepYLCV, chili mutant lines also showed morpho-agronomic variability during the field experiment. The results of variance analysis for all morpho-agronomic characters are shown in Table 3. Significant effects of genotype were observed for all traits under study, except for the number of branch nodes and length of fruit. Genetic variability plays important in crop improvement. The variability that is observed in the basic population is the chance of improvement. The highly significant difference among the mutants for many characters indicated the existence of large genetic variability among the genotypes. These results are in good agreement with some earlier findings of genetic variability of EMS-induced mutagenesis [33–37].

3.2.3. Estimation of chili mutant lines genetic parameters. Table 4 showed estimation of genotypic (Vg) and phenotypic variances (Vp), GCV, PCV and h^2. High Vg and Vp were recorded for plant height (86.01 and 126.48 cm) and harvest period (64.19 and 72.23 days), respectively. The low values of Vg and Vp were observed for the number of branch nodes, weight per fruit, length of fruit, fruit diameter, and length of fruit stalk. In general, the Vps were higher than Vg for all the characters. GCV and PCV for the number of fruits both were 0.00%, whereas for the number of branch nodes were 12.68 and 30.36%, respectively. According to Deshmukh [22], PCV and GCV values greater than 20% are regarded as high and values between 10 and 20% to be medium, whereas values less than 10% are considered to be low. Accordingly, high PCV and GCV were recorded for dichotomous and harvest period, whereas traits with moderate PCV and GCV were height of plant and width of canopy.

The number of branch node had high PCV and medium GCV. Traits having low PCV and GCV were the number of fruits per plant, weight per fruit, length of fruit, diameter of fruit, length of fruit stalk, and weight of seed per plant. High values of PCV and GCV indicated the existence of substantial variability for such characters and selection might be effective based on these characters. A similar finding was reported by earlier researchers for dichotomus and plant height [38]. In this study, the PCV was relatively greater than GCV for all traits; indicating a high contribution of genotypic effect for phenotypic expression of such characters.

Table 2. List of chili mutant lines resistant to PepYLCV under natural infection during the dry season of 2016 in Lembang.

Mutant lines	Disease incidence (%)	PCR	Resistance status	Mutant lines	Disease incidence (%)	PCR	Resistance status
M.25/M3.613.2	45.33	+	S	M3.149.3	20.00	+	S
M.33/M.123.3	5.33	-	R	M3.153.1	1.33	+	S
M.34/M.114.1	14.67	+	S	M3.153.2	8.00	+	S
M.36/M3.114.3	54.67	+	S	M3.167	0.00	-	R
M38/M3.148.2	65.33	+	S	M3.168	0.00	-	R
M.43/M3.139.2	22.67	+	S	M3.176	0.00	-	R
M.44/M3.139.3	4.00	-	R	M3.190	0.00	-	R
M.55/M3.201.2	8.00	+	S	M3.192	0.00	-	R
M.57/M3.801.3	24.00	+	S	M3.200.2	8.00	+	S
M. 58/M3.167.2	30.67	+	S	M3.238.1	9.33	+	S
M.60/M3.167.4	5.33	+	S	M3.238.2	24.00	+	S
M.62/M3.170.3	6.67	+	S	M3.353.1	5.33	-	R
M.63/M3.108.1	0.00	-	R	M3.420.1	26.67	+	S
M.69/M3.200.1	0.00	-	R	M3.517.1	20.00	+	S
M.74/M3.108.3	5.33	+	S	M3.517.2	33.33	+	S
M.76/M3.160.2	4.00	-	R	M3.711.1	20.00	+	S
M.77/M3.160.3	10.67	+	S	M3.711.2	37.33	+	S
M.82/M3.146.1	0.00	-	R	M3.711.3	24.00	+	S
M.84/M3.149.1	0.00	-	R	M3.801.1	12.00	+	S
M.86/M3.123.1	0.00	-	R	M3.801.2	33.33	+	S
M.87/M3.100.2	0.00	-	R	M3.902.1	34.67	+	S
M3.113.3	9.33	+	S	M.902.3	28.00	+	S
M3.122.1	21.33	+	S	Gelora	34.67	+	S
M3.138	6.67	+	S	Chiko	32.00	+	S
M3.148.1	13.33	+	S	Tanjung-2	42.67	+	S

S = susceptible, R = resistant.

Table 3. Mean square of combined analysis for all the characters studied of 47 chili mutant lines of Gelora cultivar and three genotype controls (Gelora, Chiko and Tanjung-2).

Source	Df	Dic	HP	WC	NBN	HvP	NF	WF	LF	FD	LFS	WS
Replication	2	0.904	56.792	0.102	17.846**	31.927	2.69	15.762	50.22	0.006**	0.004	0.058
Mutant	49	113.93**	301.49*	81.63**	0.201	200.60**	1,011.71**	3.11*	58.08	0.10**	0.37**	11.76**
Error	98	1.73	43.469	0.096	0.549	8.047	1.92	0.376	50.53	0.002	0.013	0.016

*Significantly different at 5% level.
**Significantly different at 1% level.
Df = degres of freedom, Dic = dichotomus, HP = height of plant, WC = width of canopy, NBN = number of branch nodes, HvP = harvest period, NF = number of fruits, WF = weight per fruits, LF = length of fruit, FD = fruit diameter, LFS = length of fruit stalk, WS = weight of seed.

Estimates of heritability in broad-sense ranged from 17.9% for the number of branch node to 99.7% for the width of canopy (Table 3). Heritability values greater than 80% were very high, values from 60–79% were moderately high, values from 40–59% were medium and values less than 40% were low [21]. Accordingly, very high heritability was shown by the width of canopy, weight of seed, number of fruit per plant, length of fruit stalk, dichotomous, and harvest period. The higher values of heritability estimations of traits are indicators of a greater proportion of genetic components in relation to the environment [39], and selection for such characters could be fairly easy due to high additive effect [40]. High estimates of broad-sense heritability have also been reported by previous researchers for days to first harvest and fruit length [41], the number of fruit per plant [42] and canopy diameter [43].

Table 4. Genotypic (Vg) and phenotypic (Vp) variances, genotypic (GCV) and phenotypic (PCV) coefficient of variances and broad-sense heritability (h^2) for all traits of Gelora cultivar of *Capsicum annuum*.

Character[a]	Min	Mean	Max	Vg	Vp	GCV (%)	PCV (%)	h^2 (%)	GA	GAM
Dic (cm)	10.55	30.2	32.42	37.40	39.13	20.25	20.71	95.6	12.32	40.7
HP (cm)	34.30	57.2	75.00	86.01	129.48	16.21	19.88	66.4	15.56	27.2
WC (cm)	29.90	33.9	49.87	27.18	27.27	15.35	15.38	99.7	10.73	31.6
NBN	2.40	2.7	3.67	0.12	0.67	12.68	30.36	17.9	0.3	11.1
HvP (days)	24.00	38.8	54.00	64.19	72.23	20.66	21.92	88.9	15.56	40.1
NF	22.33	50.3	71.00	18.35	18.42	0.00	0.00	99.6	8.81	17.5
WF (g)	2.73	4.4	8.47	0.95	4.08	0.01	0.04	23.3	0.97	22.0
LF (cm)	3.86	9.2	11.67	1.59	7.26	0.00	0.02	21.9	1.22	13.3
FD (cm)	0.78	1.1	1.93	0.04	0.09	0.01	0.01	44.4	0.27	24.5
LFS (cm)	2.38	3.0	3.53	0.34	0.35	0.01	0.01	97.1	1.18	39.3
WS (g) per plant	1.13	4.6	7.27	1.98	1.99	0.02	0.02	99.5	2.89	62.8

[a] See Table 3 for explanation of the abbreviations.

The estimated GA values for all characters are presented in Table 4. Due to the different scale of traits, GA was calculated as percentages of GAM. High heritability, along with high GA is an important factor for predicting the resultant effect for selecting the best individuals [44]. Therefore, genetic advance gives an idea of possible improvement of the new population through selections. The genetic gain depends on the amount of genetic variability and magnitude of the masking effect of the environment. Values of genetic advance as a percentage of mean ranged from 11.1 to 62.8. High values of genetic advance as percentage of mean estimates coupled with high estimates of heritability expected in weight of seeds per plant indicate the preponderance of additive gene action for the

expression of these traits [39]. The number of branch node exhibited low heritability and genetic advance, indicating that this trait is controlled by non-additive genetic effects.

4. Conclusions

EMS mutagenesis successfully induced genetic variability in the Gelora cultivar of *C. annuum*. Resistant chili pepper mutant lines against PepYLCV were identified from M_2 generation in a greenhouse screening and verified in a field experiment. The resistant mutant may be used as genetic material to identify the genes controlling resistance against PepYLCV, which is a commercially desirable trait. Chili pepper mutant lines also showed genetic variation in morpho-agronomic and yield component. Some of morpho-agronomic and yield component traits could be used as useful criteria for selection in the chili improvement, due to high variation in genotypic coefficient, heritability estimation, and genetic advance value. Further work is needed to analyze these mutants and determine the genetic reasons underlying the morphological changes in order to genetically improve *C.annuum* cultivars.

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