Development of Bottle Gourd Lines Resistant to Zucchini Yellow Mosaic Virus Using Ethyl Methanesulfonate Mutagenesis

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Abstract. Zucchini yellow mosaic virus (ZYMV) causes serious damage to cucurbit crops worldwide and can be spread by aphids, by mechanical injury, and in seeds. With the popularization of cucurbit grafting, the use of susceptible rootstock has increased the risk of ZYMV infection in cucurbit crops. In China, the bottle gourd (*Lagenaria siceraria*) is a widely used rootstock in grafted watermelon production. However, few resistant bottle gourds are available commercially. This study developed bottle gourd lines resistant to ZYMV using ethyl methanesulfonate (EMS) mutagenesis. A new mutated bottle gourd population (M₁) was generated by treating seeds with EMS. Diverse phenotypes were observed in the seedlings, flowers, and fruit of M₂ plants, some of which are of potential commercial interest, such as dwarfing and different fruit shapes. Based on the M₂ phenotypes, 106 M₃ lines were selected and screened for resistance to ZYMV by mechanical inoculation and agroinfiltration. Nine M₃ lines were resistant to ZYMV during three tests. One inbred M₄ line (177-8) was developed and showed stable resistance and no virus when tested using a double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and polymerase chain reaction. These resistant lines are promising materials for developing watermelon rootstock and exploring resistance genes as new ZYMV-resistant resources. EMS induction could be a practical strategy for creating resistant cucurbit crops.

Zucchini yellow mosaic virus (ZYMV), a member of the genus *Potyvirus* in the family *Potyviridae*, infects cucurbits worldwide. Plants infected with ZYMV develop blistering and malformed leaves. The disease can reduce fruit yield and quality, thereby destroying the crop (Desbiez and Lecoq, 1997; Nagendran et al., 2017). It can be transmitted mechanically or by aphids using a nonpersistent mode,

as well as by seeds (Desbiez and Lecoq, 1997; Simmons et al., 2013). Considerable efforts have been devoted to controlling ZYMV disease, including cross-protection (Gal-On, 2000; Wang et al., 1991), using chemicals to control vectors, host resistance (Guner et al., 2018), and biological control. However, ZYMV is prevalent worldwide. An economical and efficient way of solving this problem is breeding resistant varieties. However, the lack of resistant material limits progress in breeding resistance.

Grafting technology is increasingly being used in vegetable production to allow continuous cropping (Colla et al., 2010). For cucurbits, the bottle gourd (*Lagenaria siceraria*) is an essential rootstock used to improve the disease resistance of grafted plants (also known as a scion), especially watermelon or melon (Gaion et al., 2017). However, the scion might be incompatible or potentially infected with seed-borne viruses during the grafting process. Natural infection of the bottle gourd by ZYMV has been reported in

many countries (Fidan et al., 2016; Svoboda et al., 2013). Susceptible rootstock increases the risk of yield loss of watermelon and melon production. With the popularization of watermelon and melon grafting, the demand for disease-resistant rootstock has increased markedly. However, commercial bottle gourd cultivars are generally susceptible to viral diseases (Ling et al., 2013). Compared with conventional breeding, mutation breeding creates variability in a crop species and shortens the time taken to develop cultivars. EMS is a mutagenic agent that causes random point mutations at high density and can cause allelic mutations throughout the genome of any species (Okagaki et al., 1991). EMS mutagenesis to generate resistance to potyviruses has been reported for *Arabidopsis* (Duprat et al., 2002; Lellis et al., 2002). Several Cucurbitaceae species have been subject to EMS mutagenesis, including *Cucumis melo* (Dahmani-Mardas et al., 2010; González et al., 2011), *Cucumis sativus* (Boualem et al., 2014), and *Cucurbita pepo* (Vicente-Dólera et al., 2014). Using EMS to induce virus resistance in the bottle gourd has not been reported. We developed bottle gourd lines resistant to ZYMV using an EMS-mutagenized population to produce novel material for breeding resistant cultivars or rootstock.

Materials and Methods

Derivation of M₃ bottle gourd lines. The seeds of wild-type bottle gourd ‘Anshenghulu’, an inbred line that has been self-pollinated for many generations, were provided by Ansheng Seed Company (Anhui Province, China). This variety is widely used as rootstock to graft watermelon in China because of its high affinity. Unfortunately, it is susceptible to many viruses, including ZYMV.

Approximately 11,000 Anshenghulu seeds were cracked and soaked in ≈55 °C water for 4 h. Subsequently, the seeds were divided into two equal parts and treated with 1.2% and 1.5% (v/v) EMS (Aladdin, Shanghai), respectively, at 25 ± 2 °C for 8 h, which we previously determined was the optimal semi-lethal condition (Kang et al., 2017). After treatment, the M₁ seeds were sown in a field. At flowering, the plants self-pollinated; ultimately, 364 M₂ families were harvested. Approximately 10 to 20 seeds from each M₂ family were selected, grown, and self-pollinated. Finally, 2444 M₃ lines from 303 M₂ families were obtained and used to screen for resistance to ZYMV.

Phenotyping M₂ individuals. During growth of the M₂ plants, phenotypic variation in seedlings, leaves, flowers, and fruit was evaluated. Viral infections caused by aphids and seeds were observed in M₂ plants in the field. At the flowering stage, we also recorded the M₂ families with no viral symptoms, such as mosaic, mottle, yellowing, and others, which could indicate resistance to the virus.

Inoculum preparation and inoculation. The ZYMV isolate (ZYMV-CH87) originated from a naturally infected melon and was stored at –80 °C. Before inoculation, the virus isolate was maintained on susceptible zucchini (*Cucurbita pepo* L.) cultivars. For

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mechanical inoculation, an inoculum was prepared by homogenizing leaves of infected zucchini in 0.01 M phosphate buffer (pH 7.2; 1:10 w/v) using a mortar and pestle. Seedlings were inoculated at the one true leaf stage by mechanically injuring seedling cotyledons using carborundum powder and then gently rubbing the inoculum by hand to ensure that they were coated with the homogenate. The inoculum was kept on ice until inoculation was complete. After inoculation, the carborundum was rinsed from the leaves.

Recently, our group (Liu et al., 2020) constructed an enhanced green fluorescent protein (eGFP)-carrying infectious clone of ZYMV-CH87 (named pXT1-ZYMV-eGFP) and used it to inoculate plants by agroinfiltration. *Agrobacterium tumefaciens* strain GV3101 harboring pXT1-ZYMV-eGFP was cultured in lysogeny broth medium containing 100 mg/L kanamycin at 28 °C overnight. Then, 1 mL of bacterial culture was added to 10 mL of lysogeny broth medium containing 100 mg/L kanamycin and 100 μ M acetosyringone and further incubated at 28 °C until the optical density at 600 nm reached 1.5. The *A. tumefaciens* cultures were centrifuged; then, the cells were resuspended in 10 mL of infiltration medium (10 mM MES, 10 mM MgCl₂, and 100 mM acetosyringone) and incubated at room temperature for 2 to 12 h. Then, the two cotyledons were infiltrated with *A. tumefaciens* suspension at the one true leaf stage.

Mechanical inoculation was used during primary screening and retesting. The method of agroinfiltration with pXT1-ZYMV-eGFP was used during the third test.

Planting and screening. The plants were grown individually in 13-cm-diameter, 11-cm-high plastic pots. During primary screening, all plants were maintained at 20 to 35 °C for 30 d in insect-proof cages to prevent contamination by other viruses. Retesting and a third test were performed after the last screening to verify the resistant lines in growth chambers under an 8-h dark (20 °C)/16-h light (30 °C) cycle. For all tests, inoculated wild-type was used as a positive control and healthy wild-type was used as a negative control. Viral symptoms were evaluated 30 d postinoculation (dpi), along with positive and negative controls. We used one replicate per line during the primary screening, three replicates each during the retest and third test, and three replicates for positive and negative controls during all tests. The presence of ZYMV in plants was assessed using a DAS-ELISA, reverse-transcription polymerase chain reaction (RT-PCR), and fluorescence of ZYMV-eGFP at 30 dpi.

Viral detection by DAS-ELISA. Young leaves were collected at 30 dpi. Extraction buffer (1:10 w/v) was added to the leaves, which were then homogenized. DAS-ELISA was performed to detect the virus with a specific commercial polyclonal antibody (Adgen, Leamington Spa, UK) according to the manufacturer's instructions. The results were regarded as positive when the ratio of (tested sample-blank)/(negative control-blank) was ≥ 3 (Chewachong et al., 2015).

Fig. 1. Examples of morphological mutants observed in the bottle gourd M₂ population grown in the greenhouse and the field. Leaves and flowers of wild type (A and B). Different shapes, colors, and leaf sizes (C–E). Mutated cotyledons (F and G). Dwarf or semi-dwarf plants with compact architecture (H and I). Variations in M₂ flowers, mainly nonpetal phenotypes (J–L).

Viral detection by RT-PCR. Total RNA was extracted from the M₃ and M₄ plants. First-strand cDNA was synthesized from 2 μ g of total RNA using a Reverse First Strand cDNA Synthesis Kit (Tiangen Biotech, Beijing, China). The primers 5'-TCGTTGCAACCGAAATTC-3' (forward) and 5'-GCCAACTCTGT-AATGCTTC-3' (reverse) were designed from the ZYMV genome sequence. PCR was performed using these primers to detect the virus with an initial incubation at 95 °C for 5 min, 38 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 90 s, and a final 5-min extension at 72 °C. The PCR products were detected by 1% agarose gel electrophoresis.

Observation of ZYMV-eGFP fluorescence. New leaves were collected to observe GFP fluorescence by confocal microscopy (Leica TCS SP5; Leica Camera AG, Wetzlar, Germany) at 30 dpi. For simultaneous imaging, GFP fluorescence was excited at the 488-

nm laser line. The detection spectrum range was 500 to 587 nm.

Results

Phenotyping the mutagenized population.

To produce M₃ seed stocks, 10 to 20 seeds each of the 364 M₂ families were sown in a nursery and grown to fruit maturity in an open field; then, the M₃ seeds were harvested from individual M₂ plants. As a result, 2444 M₃ lines from 303 M₂ families were collected; however, 61 M₂ families died during growth or did not produce M₃ seeds. At least 10 plants per M₂ family were scored for visual phenotype changes in seedlings, flowers, and fruit at key development stages from germination until fruit maturation. Compared with untreated plants, $\approx 4.6\%$ of the M₂ families showed morphological changes, including leaves of different shapes, colors, and sizes, mutated

Fig. 2. Diverse shapes and colors of M₂ fruits grown in the field. (A) Wild type. (B–K) Variants. (H–K) Observations of one M₂ family.

cotyledons, and dwarfing (Fig. 1A and C–I). Flower variation, mainly in the nonpetal flowers, occurred in 3% of the M₂ families (Fig. 1B and J–L). More than 3.9% of the M₂ families exhibited variation in fruit shape and color (Fig. 2). Overall, 11% of the M₂ families showed at least one visible mutant trait.

Additionally, 38 M₂ families showed no viral symptoms with natural infection, whereas in other M₂ families at least one plant displayed viral symptoms. The infected virus was confirmed using RT-PCR (Supplemental Fig. 1), including, but not limited to, ZYMV, watermelon mosaic virus (WMV), cucumber green mottle mosaic virus (CGMMV), and squash mosaic virus (SqMV).

To screen for resistance to ZYMV, 106 M₃ lines were selected, including 10 lines from 10 M₂ families with no viral symptoms in the open field, six lines from five M₂ families with mutated flowers or fruits, and five lines from three M₂ families with dwarfing. The other lines were from M₂ families with normal visual phenotypes (Table 1).

Primary screening. During this test, a total of 106 M₃ lines were inoculated mechanically and screened. Plants were classified as susceptible depending on foliar symptoms and the ELISA results at 30 dpi. At 10 dpi, mosaic symptoms were observed on the wild type and some lines. By 30 dpi, 29 lines showed viral symptoms that ranged from very mild to severe mottled mosaic, yellowing, chlorosis, and chlorotic or necrotic spots, whereas severe yellowing mosaic with chlorotic spots was observed on the wild type (Fig. 3). The DAS-ELISA confirmed the presence of ZYMV virus in the 29 symptomatic lines. No obvious viral symptoms were observed in the other 77 lines.

Retest. The retest of 77 asymptomatic lines and the wild type through mechanical inoculation showed that there were escapes during primary screening. Forty-four lines were identified as resistant during primary screening, but they were susceptible according to the retest at 30 dpi. Their susceptibilities were also determined by DAS-ELISA or RT-PCR. Two lines (172-3 and 338-12) did not show systemic symptoms at 30 dpi; they either expressed symptoms solely on the initial leaves or expressed no clear symptoms, although the virus was detected in young systemic leaves. This suggested that the two lines recovered from the infection.

Third test. To decrease the possibility of escapes, during the third test, an infectious clone of ZYMV carrying eGFP was used to inoculate the wild type and 33 lines that were asymptomatic during the retest. Again, all inoculated wild-type plants (three replicates) showed viral symptoms at 10 dpi and subsequently developed severe yellowing mosaic. Seventeen lines showed viral symptoms at 30 dpi and were confirmed to be susceptible by positive ELISA reactions and strong fluorescence signals. This suggested that there were also escapes during the retest study. Of the remaining 16 symptomless lines, weak fluorescence signals were detected only in vein regions in seven lines, but the ELISA results were negative; therefore, they were

Table 1. M₂ phenotypes for tested M₃ lines.

No. of tested M ₃ lines	Notes of their M ₂ families	No. of resistant lines
10	No viral symptoms in open field	1
4	With mutated flowers	0
2	With changed fruit shape	0
2	With dwarfing	1
2	With deformed leaves	2
1	With yellowing leaves	0
85	With visual normal phenotypes	5
Total: 106		9

considered moderately resistant. Ultimately, nine lines showed resistance to ZYMV for seven replicates during the three tests based on the negative ELISA results and the fluorescence signal (Table 2; Fig. 4).

Of these resistant M₃ lines, 177-8 was from an M₂ family that had no viral symptoms when growing in the open field. The M₂ families of 163-3 and 178-6 had deformed leaves, and line 008-6 was a dwarf. The phenotypes suggested that these lines had undergone additional genetic changes with EMS exposure. The other lines were morphologically similar to the wild type and fruit phenotypes.

Developed resistant line. An inbred M₄ line of 177-8 was developed and its resistance stability was tested in growth chambers by mechanical inoculation. A total of 39 inoculated plants of this line were asymptomatic (Fig. 5) and negative for ZYMV according to ELISA and PCR test results, which were consistent

with those of the M₃ line. No susceptible plants segregated during M₄ generation suggested that 177-8 line had stable resistance to ZYMV.

Discussion

Successful mutation breeding depends on the methods used, effective screening techniques, and the populations grown in M₁ and successive generations (Toker et al., 2007). For bottle gourd mutagenesis, the EMS concentrations were higher than those used for other cucurbits (González et al., 2011; Vicente-Dólera et al., 2014). This led to a high proportion (≈63%) of seedless fruit, which resulted in a small M₂ population. In *C. pepo*, 621 M₃ families were obtained when using 40 mM EMS, whereas only 95 were obtained with 80 mM EMS (Vicente-Dólera et al., 2014). However, the mutation density increases with the EMS concentration, with more multiple

Fig. 3. The expression of viral symptoms on some different lines at 30 d postinoculation (dpi) with zucchini yellow mosaic virus (ZYMV) induced by a mechanical method. (A and B) Severe yellowing mosaic with chlorotic spots. (C and D) Severe mottled mosaic. (E and F) Mild mosaic. (G) Very mild mottled chlorosis. (H) No symptoms. (I) Severe yellowing mosaic with chlorotic spots on wild type.

Table 2. Results of the third test after inoculation with the infectious clone of zucchini yellow mosaic virus (ZYMV) enhanced green fluorescent protein (eGFP).

Line	Third test at 30 dpi				Type of resistance
	Symptoms	ELISA	Fluorescence		
008-6	NS	—	—		R
010-5	NS	—	—		R
078-8	NS	—	—		R
163-3	NS	—	—		R
177-8	NS	—	—		R
178-6	NS	—	—		R
196-13	NS	—	—		R
410-2	NS	—	—		R
422-3	NS	—	—		R
095-1	NS	—	+		MR
096-6	NS	—	+		MR
124-7	NS	—	+		MR
253-8	NS	—	+		MR
255-3	NS	—	+		MR
286-9	NS	—	+		MR
337-5	NS	—	+		MR
148-3	VM.MCH	+	++		S
203-2	VM.MCH	+	++		S
331-3	VM.MCH	+	++		S
016-13	M.MCH	+	++		S
105-5	M.MCH	+	++		S
130-2	M.MM	+	++		S
163-5	M.MCH	+	++		S
177-1	M.MM	+	++		S
249-6	M.MCH	+	++		S
294-6	M.MCH	+	++		S
302-11	M.MCH	+	++		S
312-6	M.MCH	+	++		S
316-8	M.MM	+	++		S
325-10	M.MCH	+	++		S
329-5	M.MCH	+	++		S
038-7	S.YM	+	++		S
189-4	S.YM	+	++		S
CK+	S.YMCH	+	++		S
CK—	H	—	—		

NS = no symptom; VM.MCH = very mild mottled chlorotic; M.MCH = mild mottled chlorotic; M.MM = mild mottled mosaic; S.YM = severe yellowing mosaic; S.YMCH = severe yellowing mosaic with chlorotic spots; R = resistant; MR = moderately resistant; S = susceptible; H = healthy; CK+ and CK— = positive and negative controls for WT; — = negative reaction; +++ = the positive reaction of ELISA and fluorescence, the number of “+” represented roughly the signal intensity of fluorescence.

mutations (Martin et al., 2009; Minoia et al., 2010). During this study, three identified lines (163-3, 178-6, and 008-7) had multiple phenotypes combining dwarfing or deformed leaves with resistance; this might have been because of the high EMS dose. For screening, agroinfiltration was used during the third test to ensure that sufficient virus entered the plants because escape often occurs with low inoculum densities or heterogeneous environmental conditions (Guner et al., 2019).

Mutation breeding induces recessive genes more often than dominant genes. Potyvirus resistance is often conferred by recessive genes (Amano et al., 2013; Ling et al., 2009; Wang and Krishnaswamy, 2012). Many reports have suggested that mutation breeding is an efficient way to produce virus resistance with EMS treatment in plants (Manzila and Priyatno, 2020; Souza et al., 2017; Yamanaka et al., 2002). In general, mutations are beneficial with very low frequencies, but the mutation rate varies among species, traits, and even varieties of same the species (Toker et al., 2007; Wani et al., 2014). For tomato, 39% of M₂ plants showed at least one visible mutant trait, and 37% of these lines showed multiple phenotypes (Minoia et al., 2010). During our

study, 11% of M₂ plants had visible mutant traits and 8% were resistant to ZYMV. These results show the high efficiency of EMS for inducing resistance to ZYMV. Ling and Levi (2007) reported that out of 190 bottle gourd germplasms, 36 accessions had complete resistance to ZYMV and 64 had incomplete resistance. Perhaps the bottle gourd has many potential resistance genes. The lines identified in this report are valuable for further exploration of resistance genes.

The bottle gourd is the preferred rootstock for watermelon because it controls soil-borne diseases and has no effect on fruit quality (Davis et al., 2008; Fidan et al., 2016). Despite a long cultivation history, there are no virus-resistant bottle gourd cultivars. The resistant lines identified during this study may be useful as watermelon rootstock or valuable for breeding, exploring resistance genes, and characterizing traits.

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Fig. 4. Detection of the GFP signal in leaves at 30 dpi with zucchini yellow mosaic virus (ZYMV) enhanced green fluorescent protein (eGFP) by agroinfiltration. The left row suggests the symptoms of detected lines. Strong fluorescence signals are detected in lines with severe symptoms (A; wide-type), mild symptoms (B; 177-1), and recovery (C; 016-13). Weak fluorescence signals are detected in vein regions (D and E; 124-7 and 253-8) in some symptomless lines. No fluorescence signals are detected in some symptomless lines (F; 010-5).

Fig. 5. No symptoms in 177-8 M₄ plants at 30 d postinoculation (dpi) with zucchini yellow mosaic virus (ZYMV) induced by a mechanical method in growth chambers.

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Supplemental Fig. 1. The results of one-step reverse-transcription polymerase chain reaction (RT-PCR) to detect squash mosaic virus (SqMV), cucumber mosaic virus CMV, zucchini yellow mosaic virus (ZYMV), watermelon mosaic virus (WMV), and cucumber green mottle mosaic virus (CGMMV) in M_2 plants in the field. T = tested sample comprising a mixture of RNAs from 14 M_2 plants with virus-like symptoms in the field; N = negative sample of a healthy plant; M = marker. The middle line with Cp-1, Cp-2, etc., indicates the specific primers for viruses. Except for SqMV and CGMMV, other viruses were detected using two different specific pairs of primers. The bands marked with a red arrow indicate positive results.