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Development of cucumber lines resistant to *Cucumber mosaic virus* by ovule culture

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One-hundred varieties of cucumber from different origins: China, India, Japan, Malaysia, Pakistan, Philippines, Thailand and the USA were screened for resistance to cucumber mosaic virus by mechanical inoculation. Twenty cucumber seedlings were planted in seed trays and kept under greenhouse conditions. Seedlings at the cotyledon stage were inoculated with virulent CMV subgroup I isolates collected from different cucumber plantations. Leaf samples were collected 4 weeks after inoculation and the DAS-ELISA was used for virus determination. The results revealed that the majority of the tested lines showed different symptoms ranging from severe to mild mosaic and some with no symptom which corresponded to the DAS-ELISA reading. Forty two selected lines were used for further selfing and tested in the S_1 generation. Five lines showing no symptom were used for double haploid plant production. Five lines of double haploid plants obtained from ovule culture were mechanically inoculated with CMV and evaluated for their level of CMV resistance by DAS-ELISA. The double haploid plants produced were highly resistant to the virus as was suggested by the first screening. All of resistant lines were selfed and seed was harvested.

Keywords: CMV resistance, cucumber screening, DAS-ELISA, ovule culture, double haploid cucumber

Introduction

Cucumber)*Cucumis sativus* (is one of the most economically important vegetable crops in Thailand. Cucumbers grown in the fields are seriously affected by a number of diseases caused by fungi, bacteria, phytoplasmas, nematodes and viruses) Jarvis, 1992(. Cucumber mosaic virus)CMV (is one of the important viruses causing yield losses as high as 40-60%)Varma and Giri

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1998 .(Most of the commercial cultivars of cucumber are susceptible to this virus and when infected show mosaic, mottling and distortion of leave and fruits. CMV is one of the most wide spread viruses in the world infecting over 1200 species belonging to more than 100 families of dicotyledonous and monocotyledonous plants (Edwardson and Christie, 1991 Rossinck, 2002). The virus is transmissible in the laboratory by mechanical inoculation with plant sap and naturally transmitted by more than 80 species of aphids in a noncirculative manner (Palukaitis and Garcia-Arenal, 2003). Due to its wide host range, rapid spread by vectors and lack of suitable host resistance, management of CMV is difficult by cultural practices alone)Munshi *et al*., 2008 .(The purposes of this study were to screen cucumber cultivars resistance to CMV, and to establish double haploid plant lines by ovule culture which could be used in a cucumber-breeding program in order to facilitate the development of new virus resistant commercial varieties.

Materials and methods

Screening of cucumber accessions for CMV resistance

One-hundred accessions of cucumber from different sources: China, India, Japan, Malaysia, Pakistan, Philippines, Thailand and USA were screened for the CMV resistance. Twenty cucumber seeds of each accession were sown in seed trays and kept under greenhouse conditions. The leaves of seedlings at the cotyledon stage were inoculated with the virulent CMV I (HC-53). After 10mins, the inoculated leaves were rinsed with tap water and the plants were maintained in a greenhouse that used netting to exclude insects. Plants were monitored for symptom expression and leaf samples were collected 4 weeks after inoculation. DAS-ELISA (Agdia, Elkhart, IN, USA) was used for virus determination according to the manufacturer's guidelines. Resistance lines were used for selfing schemes.

Double haploid production

Five accessions of CMV tolerant cucumber from Thailand were sown in seed trays. Seedlings at the cotyledon stage were transferred to 10.5 in diam. pots and maintained in an insect-exclusion greenhouse. Petals and styles of ovaries at 1 d before anthesis were removed, the ovaries were surface sterilized twice with 10 % calcium hypochlorite and 3% sodium hypochlorite for 20 mins and rinsed three times with sterile distilled water. Ovaries were sliced in a lamina air flow cabinet, and then transferred to the Cucumber Basal Medium supplemented with 5 ppm $AgNO_3$ for 1 month. Ovules were then transferred to

induction medium supplemented with kinetin, 6-benzylaminopurine (BA)/ 6-(gamma, gamma-Dimethylallylamino) purine (2ip): IAA at ratios 2:1, 3:1 and 4:1 ppm and grown for 2 months. The green embryos were transferred to regeneration medium supplemented with BA/2ip: IAA at ratio2:1, 3:1, 4:1 and 5:1 ppm. The cultures were incubated at 25 ^{o} C with a 16h day and 8h night regime. The regenerants were cultured on MS medium for rooting. The chromosome numbers of the regenerants were measured by direct chromosome counting from either the root tips or the tendril cells. The haploid plants (n = 7) were treated with 400 ppm colchicines for 40 mins. and cultured on the same medium. The dihaploid plants (2n = 14) derived from the colchicines treatment and the auto-dihaploid plants from the culture were selected and used for further CMV resistance screening.

Screening of CMV resistant double haploid lines

Plantlets of double haploid (DH) lines plantlets which obtained from ovaries cultured were multiplied in the laboratory after that were transplanted and grown in the 25 cm. pots under the insect exclusion greenhouse. Nine clones of DH at the 4-5 leaf stage were inoculated with virulent CMV by rubbing the inoculums) 1:5 dilution leaf material: 0.1 M phosphate buffer pH 7(on carborundum dusted leaves (Figure 1). After 15 min, the inoculated leaves were rinsed with tap water and kept under the insect exclusion greenhouse. Inoculated plants were monitored for 4 weeks after inoculation: symptoms and disease severity were recorded and plants were tested for CMV using DAS-ELISA.

Fig. 1. Double haploid at the stage that was used for CMV I resistant screening

Results and discussions

Screening of CMV resistant cucumber lines

After inoculation with the CMV I (HC-53), some cucumber accessions showed different symptoms ranging from mosaic, mottling, vein banding, and vein clearing, while others were symptomless. Based on symptom expression following inoculation with CMV, the cucumber accessions clustered into two major groups: forty-four accessions were scored as highly resistance, and fifty-six were identified as susceptible (Table 1).

Table 1. Reaction of cucumber lines to mechanical inoculation with CMV

Countries Origin	**Reaction**		**Total**
	Resistant	Susceptible	
America	5	3	8
India	2	0	2
Japan	1	3	4
Pakistan	0	1	1
Malaysia	0	1	1
Netherland	0	1	1
Philippines	1	0	1
China	6	0	6
Thailand	29	47	76
Total	44	56	100

ELISA reading: R $\leq$ 0.23 MR $\geq$ 0.24 - $\leq$ 0.45 S $\geq$ 0.46; Healthy plant = 0.21

Nine cucumbers from India (2), Phillippines (1) and six long type cucumbers (China) appeared to be highly resistant to the CMV (HC-53) by showing ELISA values less than 0.23. Especially, six accessions from China used in this study which were agreed with Heyvey (1997) who reported that Chinese long green cucumber varieties were resistant to CMV. However, genetic and molecular characterization of the resistant accessions is needed.

Double haploid production

After sterilization with the surface disinfectants, thin slices of ovaries from the selected five accessions were cultured on an induction medium for 15 – 30 days based on the genotypes of the cucumbers then transferred to a differentiation medium for the further development. One week after culture, growth of ovules protruding from the slices was noticeable, and globular-stage embryos formed (Figures.2A and B(. The tissues were subsequently transferred to MS plus BAP and IAA at a ratio 2:1 for the further regeneration. The first

visual shoot-like organogenesis, which appeared as cotyledon-like structures (Figure. 2C) were found in the second week after culturing on the medium.

New shoot formation, mostly two-five elongated ones, developed from one callus (Figure. 2D). These plantlets rooted after being transferred to MS medium. Nine clones from five accessions were obtained, one clone from CSL 0006, five clones from CSL 0011 and three clones from lines CSL 0021, CSL 0052 and CSL 0094)Table 2(.

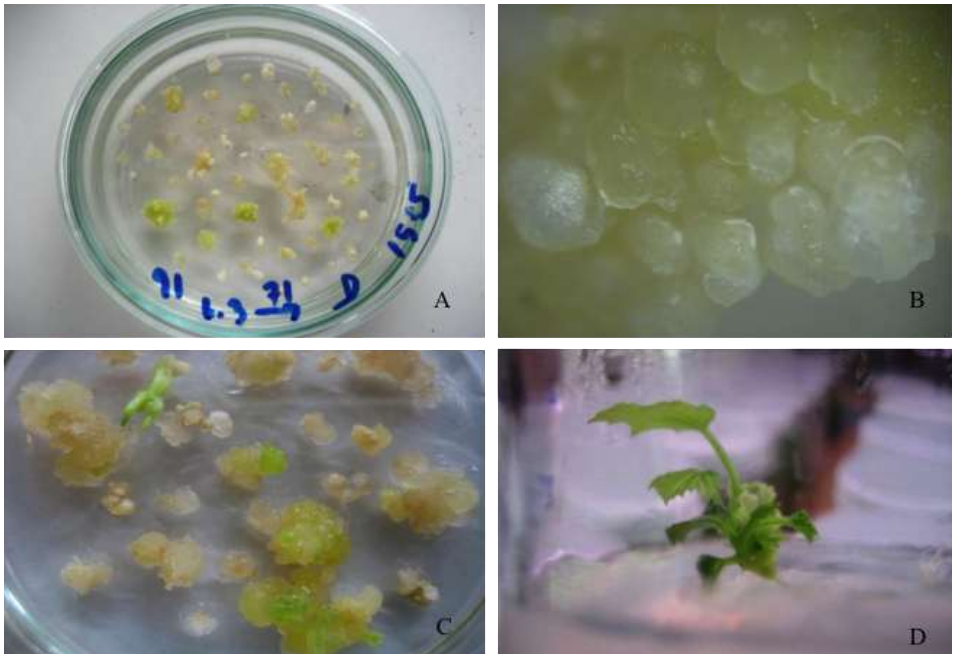

Fig. 2. Embryogenesis and regeneration of plantlets derived from ovule culture
A-B =sprouting embryo, C=shoot-like organogenesis, D= regenerated plantlets.

Table 2. Number of clones derived from different cucumber accessions induced by different hormones ratios used during induction and regeneration stages

Accessions	**Code**	**Induction stage)mg/L(**	**Regeneration stage) mg/L(**	**Number of clone(s)**
CSL 0006	86S2	BA;IAA)2:1(	2ip: IAA)5;1(	1
CSL 0011	91	BA; IAA)2:1(	BA: IAA)2:1(	5
CSL 0021	101	BA:IAA)4:1(	BA: IAA)4:1(	1
CSL 0052	132 S2	BA :IAA)8:1(	kinetin 0.5mg	1
CSL 0056	174	BA;IAA)3.5,1(	BA: IAA)5:1(	1

Each variety responded differently to the types and ratio of hormone concentrations. AbdEl-Maksoud *et al*. (2009) have reported that the media and suitable hormone balance were key factors affecting the success of anther culture of the different cucumber genotypes. In this study, induction and regeneration medium, MS supplementary BAP and IAA ratio 2:1 was the most suitable medium for embryogenesis and regeneration of 5 clones from accession CSL0011, however different types and concentrations of hormones for embryogenesis in other cucurbits were reported. Metwally et al., (1998a) used Murashike and Skoog (MS) medium supplemented with 1-5 mg dm-3 2, 4-D and 30 g dm-3 for unpollinated ovules cultured of pumpkin. Moreover Xie *et al*. (2006) applied N6 medium supplemented with 2, 4-D, naphthalene-1-acid and benzyladenine (BA) in ovules cultured of summer squash. Gémes-Juhász et al., 2002 used cucumber basal medium (CBM) supplemented with 0.02 mg dm-3 of thidiazuron and 40g dm-3 of sucrose. Besides types and concentration of hormones used in the culture media, the donor genotype was one of many factors important to unfertilized ovary/ovule culture (Chen *et al*., 2010). Gynogenesis efficiency in plants was highly dependent on the variety used. Furthermore, we found that the quality of donor materials and the growth conditions of plants were also affected on gynogenesis of ovules.

Screening of CMV resistant DH

The DH cucumbers showed different level of resistance as shown in Table 3. Two clones, 86(1) and 91(1), that showed no CMV I symptoms after 30 days after inoculation also tested negative for the virus by ELISA, compared to the healthy control plants and the negative control, and were identified as highly resistant (R) (Table 3). Four lines form code 91 (accession CSL 0011) and 1 line from code 132 (accession CSL 0052) showing mild mosaic were classified as moderately resistant (MR). Two lines from code 101 (accession CSL0021) and code $174S_2$ that showed strongly mosaic symptom were classified as susceptible.

Table 3. Reactions of DH line cucumbers resistance to CMV

Accessions	Code	DH lines obtained	Level of resistance		
			Resistant (R)	Moderately resistant (MR)	Susceptible (S)
CSL 0006	86	1	1	-	-
CSL 0011	91	5	1	4	-
CSL 0021	101	1	-	-	1
CSL 0052	132	1	-	1	-
CSL 0094	$174S_2$	1		-	1
Total		9	2	5	2

Resistance screening based on the ELISA reading: R $\leq$ 0.23; MR $\geq$ 0.24 - $\leq$ 0.45; S $\geq$ 0.46 (2x of Negative control); Healthy plant = 0.21

CMV causes very significant losses to cucurbit production worldwide. Therefore, double haploid cucumber production and screening for resistance to this virus can accelerate breeding programs through the use of homozygous double haploid lines (DHL) and facilitate the selection of desired (e.g. disease-resistant) genotypes for breeding. Lotfi *et al*. (2003) utilized hybrid melons which showed resistance to various viruses as donor to produce DH melons. Furthermore, Kuzuya *et al*. (2003) selected two lines of melons resistant to powdery mildew for the haploid plants production and found that more than half of the haploid plants displayed the same level of resistance as the donor plants. The different levels of CMV resistance observed in the DH cucumbers tested could be explained physiologically by differing resistance response capacities of the clones. The different plant virus interaction leaded to various class of resistance, factors involved in this expression based on the cultural practices of the farmers. Over nitrogen fertilizer application to the crop mostly reduce the resistant character of the plant especially in the case of virus infection (Bawden and Kassanis, 1950, Singh, 1970). Screening for the resistant lines needed to be done under the same cultural practices and environment. Resistant level also affected by the different immune expression in the host plants. Kranthi *et al*., 2013 reported that during viral infection, a hypersensitive response (HR) by the plant is an early response triggered by the host's R protein induced by the virus (Avr gene). The plant's defensive reactions include such metabolic changes as production of reactive oxygen ions as H_2O_2, O^{2-}, NO as well as salicylic acid involved in HR and signal transduction of the jasmonic acid (JA) pathway. Furthermore, during HR, vacuolar processing enzymes are activated which act as effectors of cell death or necrosis during HR)Mur *et al.*, 2008(. Various defense phenomena, resulting in different levels or tolerance, have been identified. In an immune plant, the virus is apparently unable to multiply)Loerenstein, 1972(. The DH cucumbers resistant to CMV I were

planted in an insect proof net house for seed production as well as planted in the field for natural CMV resistance screening. The resistant lines obtained from the field screening will be used for the breeding program of the seed cluster project supported by the National Science Technology and Development Agency Thailand. Future studies are needed in order to clarify the resistance mechanism of cucumber to CMV infection.

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