Journal of Applied Horticulture, 22(2): 87-91, 2020
DOI: 10.37855/jah.2020.v22i02.17
Journal of Applied Horticulture
ISSN: 0972-1045

BCAT- based marker for marker-assisted selection in Vietnam cucumber breeding

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Abstract

Yield imporovement is one of the major breeding objectives of cucumber improvement. Gynoecious, an important agricultural trait which highly correlates with yield, was proved to be controlled by F locus. Conventional plant breeding approach has some limitations in term of potential inaccuracies and time-consuming. Molecular marker-assisted breeding is, therefore, an effective alternative solution. F locus– linked molecular marker has been reported previously. The main aim of this project was to evaluate the potential applicability of this F locus–specific marker for marker–assisted selection in Vietnam cucumber breeding program. Three different cucumber populations *e.g.,* pure lines, F2 and F3 population were utilized with 13, 131 and 84 plants, respectively for each population. Plant sexual phenotypes were determined. Sequencing reactions were performed for *BCAT* 3'UTR of 3 gynoecious and 2 monoecious pure lines. Plant genotypes were determined by standard PCR with a primer pair amplifying a 56 bp-deletion region in *BCAT* 3'UTR. A 56 bp-deletion was found in *BCAT* 3'UTR of gynoecious pure lines as compared with monoecious lines. The marker based on this 56 bp-deletion region in *BCAT* 3'UTR could help to separate cucumber plants having homozygous and heterozygous sex phenotypes. The marker genotype absolutely corresponded with monoecious trait. Especially, the marker could precisely explain for 80 % gynoecious trait. The marker highly explained for Vietnam cucumber sex traits and could be applied for marker-assisted selection in cucumber breeding program in Vietnam in future.

Key words: *BCAT*, *Cucumis sativus* L, gynoecious, marker-assisted selection, co-dominant marker

Introduction

Cucumber (*Cucumis sativus* L., 2n = 14), a member of family Cucurbitaceae, is both an economically and biologically important vegetable crop worldwide. It could be eaten fresh or consumed as a processed product. Of all cucurbits, cucumber is cultivated more broadly in which its production was the second largest as compared with other vegetable species (Pitrat *et al.*, 1999).

Fruit yield, a quantitatively inherited trait, is the major breeding objective of cucumber improvement (Wehner *et al.*, 1989; Wehner, 1989; Staub *et al.*, 2008). Fruit yield is influenced by genotype and environment, and also a small interaction between genotype and environment (Wehner, 1989; Staub *et al.*, 2008). Also, yield has a low heritability (Wehner, 1989; Staub *et al.*, 2008).

Cucumber improvement by directly selecting for yield is, therefore, very difficult. In order to effectively breed for this trait, it is of importance to select traits having higher heritability and correlated well with it. These traits include the percentage of female flowers (sex expression type), stem length, number of branches per plant, fruit size, and fruit length (Wehner, 1989; Cramer and Wehner, 1998). Among these traits, sex expression has shown the potential for increasing yield of cucumber. Several studies show a positive correlation between the number of female flowers per plant and yield (Cramer and Wehner, 1998; Staub *et al.*, 2008).

Cucumbers have many sex phenotypes *e.g.,* gynoecious (pistillate flowers only), monoecious (staminate and pistillate flowers), hermaphrodite (perfect flowers), andromonoecious (staminate and perfect flowers), and androecious (staminate flowers only). These sex types are determined by three major loci *e.g.,* Female (F), Monoecious (M) and Androecious (A) (Robinson *et al.*, 1976). The F locus is responsible for female flowers development (Trebitsh *et al.*, 1997; Mibus and Tatlioglu, 2004; Knopf and Trebitsh, 2006). The M locus determines whether flowers are unisexual (M_) or bisexual (mm) (Yamasaki *et al.*, 2001; Saito *et al.*, 2007; Li *et al.*, 2009; Li *et al.*, 2012). The A locus increases male tendency if a plant is homozygous recessive aa and ff (Pierce and Wehner, 1990). The interaction of these three genes leads to the difference in sex expression of cucumber flowers *e.g.,* gynoecious (*F-M-*), androecious (*aaffM-*), hermaphrodite (*F-mm*), monoecious (*A-ffM-*) and andromonoecious (*ffmm*) (Win *et al.*, 2015).

Selection of gynoecious lines by conventional method is a time - consuming process and is based mainly on observation of flower sex expression in breeding fields. Traditional selection has some limitation *e.g.,* selection inaccuracy under field conditions as sex expression is affected by environmental factors and difficulties in early identification. Molecular maker-assisted selection of gynoecy, therefore, becomes essential to improve the efficiency and precision of conventional cucumber breeding.

Besides genetic factors, cucumber sex expression is also

influenced by environmental factors (Ikram *et al.*, 2017; Liu *et al.*, 2018). Together, these two factors mediate sex expression through changes in plant hormonal levels in which the balance between ethylene, auxins, absisic acid and gibberellins are crucial. Ethylene is considered as the primary hormone affecting the femaleness (Takahashi and Jaffe, 1984; Yamasaki *et al.*, 2003). Current theory holds that the enzyme ACC (1-aminocyclopropane-1-carboxylic acid) synthase plays a critical regulatory role in the ethylene biosynthesis (Harpaz-Saad *et al.*, 2018). In cucumber, F locus was found in long arm of chromosome 6 and contained *CsACS1* (Trebitsh *et al.*, 1997; Mibus and Tatlioglu, 2004). The dominant F allele possesses an additional copy of *CsACS1* called *CsACS1G* as compared with the recessive f allele in which only a single copy of *CsACS1* was found (Trebitsh *et al.*, 1997). *CsACS1G* has been proved to be the result of gene duplication and recombination between *CsACS1* and *CsBCAT* (Trebitsh *et al.*, 1997; Knopf and Trebitsh, 2006). Especially, the 56-bp deletion in 3'UTR of *CsBCAT* was significantly conserved polymorphism between gynoecious and monoecious cucumber lines (Win *et al.*, 2015). Therefore, the F locus-specific co-domimant molecular marker developed based on this deletion showed complete linkage to F locus and could distinguish perfectly homozygous and heterozygous gynoecious (Win *et al.*, 2015).

In this study, we exploited this F locus specific co-dominant *BCAT* marker onto Vietnam cucumber pure lines, F2, and F3 segregating populations to verify its applicability to specific identification of F locus.

Materials and methods

Plant materials and growth conditions: All cucumber lines used in this study originated from Vietnam. Plants were cultivated in net house at Tan Loc Phat Seed Limited Company from 2017 to 2018. 130 plants of 13 cucumber pure lines (Tan Loc Phat Seeds Company) were used. TLP10 (gynoecious) and TLP14 (monoecious) lines were chosen for crossing to create F1 hybrid. From the F1 generation, a single plant was self-pollinated to obtain F2. 150 individuals were randomly chosen from F2 population for testing correlation between genotype and sex phenotype. From F2 generation, gynoecious, subgynoecious, and monoecious plants (two plants per group) were self-pollinated to obtained F3. 15 plants from each group (6 groups in total) were randomly chosen for testing correlation between genotype and sex phenotype.

All plant materials used were grown in bags at day/night temperature of 24 °C/18 °C and 16 hours with assimilation light with drip irrigation of nutrient solution, following plant protection practices as necessary.

Evaluation of sex expression: The sexual phenotype of individual plants was determined by recording the sex type of all flowers in each plant every day for 8 days from the time point when the first flower appeared in the population. The plants which possess only female flowers are defined gynoecious. The plants which possess number of female flowers more than or equal to number of male flowers are defined subgynoecious. The plants which possess almost male flowers are monoecious.

DNA extraction, sequencing and genotyping: Genomic DNA was isolated from cucumber young leaves using Plant DNAzol® Reagent (Invitrogen – 10978-021) according to the manufacturer's protocol. Purified DNA was diluted to a concentration of 25 ng/µL and stored at -20 °C.

BCAT-S primer (Forward primer- CTGGACACATTTTGCA GACA; Reverse primer- TCTTCCTCAAATCCCTCGTTC) were utilized for amplifying 890 bp fragment in 3'UTR region of *BCAT*. PCR reactions were performed in a final volume of 30 µL with 0.5 µM of each primer, 50 ng of DNA, 200 µM of dNTPs, 1X of Buffer and 0.6 µL of Phire Hot Start II DNA Polymerase (ThermoFisher – F122S). Amplification was carried out as following: an initial cycle at 98 °C for 30 s, followed by 30 cycles at 98 °C for 3 s, 56 °C for 3 s and 72 °C for 7 s, and a final cycle at 72 °C for 30 s. Amplified fragments were visually analyzed after electrophoresis in 2 % agarose gel with 1X TBE buffer and stained with 0.1 µg/mL of ethidium bromide to visualize. PCR products were cleaned up and performed sequencing (3500 Genetic Analyzer, Applied Biosystem).

Cs*BCAT* primer (Forward primer -CATTGTGTGAATGAAGA CAAG; Reverse primer-CTTCAACGCAAAACCTTCATC) (Win *et al.*, 2015) were used to determine genotype for 3 surveyed populations. The reactions were performed with components as above with following cycle program: an initial cycle at 98 °C for 30 s, followed by 30 cycles at 98 °C for 3 s, 62 °C for 3 s and 72 °C for 5 s, and a final cycle at 72 °C for 30 s.

Results and discussion

Evaluation of sex phenotype of three surveyed populations: Three different cucumber populations were utilized *e.g.,* pure lines, F2 and F3 population. The first population had 13 pure lines collected from various regions of Vietnam. F2 population had 131 plants which were developed from the cross between the gynoecious TLP10 line and the monoecious TLP14 line. F2 progenies concluded 56 either gynoecious or subgynoecious plants and 75 monoecious plants. F3 population, including 6 sub-populations, were obtained by self-pollinating 6 F2 progenies. Each sub-population contained 12 to 15 plants. Four F3 sub-populations had only monoecious phenotype whilst the other two had the ratio of either gynoecious or subgynoecious to monoecious phenotypes being 8:5, and 8:7, respectively. Among the segregating surveyed populations, only 4 F3 sub-populations had sex phenotype segregated following Mendel's law of segregation (Table 1). The F2 population and the other F3 sub-populations not obeyed Mendel's law (Table 1).

Since, many reports have proved conclusively that cucumber gynoecious trait was encoded by a dominant *F* gene, the F2 and F3 progenies should have the ratio of gynoecious or subgynoecious trait to monoecious trait been around 3:1. The fact that gynoecious trait expression in some segregating populations in this study was not fitted well with this ratio could be partly explained by a relatively small number of plants utilized in each population, not fully representative for its population in general.

This study, however, attempted to properly evaluate the *BCAT* marker utility for identifying gynoecious trait in Vietnam only. Therefore, these segregating populations would seem still suitable for this aim and thus be employed for further downstream analysis.

Table 1. Evaluation of sex phenotype of three surveyed populations

Population	Total plants	Phenotype				χ^2	*P*-value
		Observed value		Expected value			
		+ Gynoecious Subgynoecious	Monoecious	+ Gynoecious Subgynoecious	Monoecious		
F1	13	5	8	-	-	-	-
F2	131	56	75	87	44	31.83	ns
F3.1	13	8	5	9	4	0.09	*
F3.2	15	8	7	10	5	0.68	*
F3.3	15	0	15	0	15	0.02	*
F3.4	15	1	14	0	15	0.02	*
F3.5	14	2	12	0	14	0.16	*
F3.6	12	1	11	0	12	0.02	*

Sex expression was determined basing on the ratio of female to male flower during 12 days from the time the first flower appeared. The significant difference between expected and observed ratio of female to male flower of each population was checked by Chi square test with df=1, ns: not significant; *: $P < 0.05$.

A 56 bp-deletion in *BCAT* 3'UTR appeared in different gynoecious Vietnam cucumber lines: It was reported that the 56 bp-deletion in *BCAT* 3'UTR was a fairly reliable marker for screening gynoecious trait in cucumber. Since, this conclusion was successfully drawn out with cucumber gynoecious lines collected from different regions around the world, whether it still remains true to Vietnam gynoecious lines needs to be conclusively proved. *BCAT* 3'UTR of 3 gynoecious and 2 monoecious pure lines were sequenced. In consistent with previous report (Win *et al.*, 2015), a 56 bp-deletion was found in *BCAT* 3'UTR of gynoecious pure lines as compared with monoecious lines, suggesting such deletion was highly conserved across diverse gynoecious lines.

56 bp- deletion in *BCAT* 3'UTR could help to distinguish between homozygous and heterozygous F locus: To determine whether *BCAT* marker could identify homozygous and heterozygous plants, a PCR primer pair which specifically amplified sequences around 56 bp-deletion region in *BCAT* 3'UTR was used. PCR products were either 160 bp or 218 bp corresponding to 56 bp- deletion (alelle F) or normal (alelle f), respectively, in *BCAT* 3'UTR.

PCR reactions were performed with DNA samples isolated from gynoecious, subgynoecious, and monoecious cucumbers. Results (Fig. 1) showed that PCR products either 160 bp *(FF*) or 216 bp (*ff)* clearly appeared. In addition, *Ff* which demonstrated by two products in a reaction, was also presented. This result suggests the 56 bp-deletion in *BCAT* 3'UTR could help to separate cucumber plants having homozygous and heterozygous sex phenotypes.

The correlation between the 56 bp- deletion in *BCAT* 3'UTR with sex traits in three different surveyed populations: To the genotypes of the three surveyed populations, PCR reactions were again performed with the primer pair amplified sequences around the 56 bp-deletion region in *BCAT* 3'UTR. Genotype of each plant in F2 and F3 populations was precisely determined. Similar to phenotype, F2 population genotype did not follow 1:2:1 ratio of Mendel's segregation law (Table 2). This could be partly explained by the small number of F2 samples which probably could not fully represent the whole population. Only two of the F3 populations had genotypes segregated following Mendel's segregation law (Table 2).

Table 2. Evaluation of sex genotypes of F2 and F3 populations

Population	Total plants	Genotype						χ^2	*P*-value
		Observed value			Expected value				
		FF	Ff	ff	FF	Ff	ff		
F2	131	38	49	44	32.75	65.5	32.75	8.86	ns
F3.1	13	7	5	1	3	6	4	5.69	*
F3.2	15	5	8	2	4	8	3	0.18	*
F3.3	15	0	0	15	0	0	15	0.02	*
F3.4	15	2	4	9	4	8	3	12.18	ns
F3.5	14	1	9	4	4	8	2	2.72	*
F3.6	12	0	6	6	3	6	3	4.21	*

Plant genotypes were determined by standard PCR with *BCAT*-based marker. The significant difference between expected and observed values of three genotypes in each population was checked by Chi square test with df=2, ns: not significant; *: $P < 0.05$

The correlation between marker genotype and sex traits of the three surveyed populations are shown in Table 3. For the pure line population, the marker correctly identified 100 % monoecious lines which could explain for 40 % gynoecious lines only. Overall, this marker could account for 54 % sex expression of the whole population. For F2 population, the correlation between genotype and sex expression of gynoecious, subgynoecious, and monoecious plants was 82, 52, and 100 %, respectively. For F3 population, the marker recognition was highest for monoecious plants (100 %), followed by the gynoecious plants (from 80 to 100 %, except a case of only 50 %), and lowest for subgynoecious plants.

Overall, through the three surveyed populations, the accuracy of the marker for determining Vietnam cucumber sexual phenotypes was tested. The marker genotype absolutely corresponded with monoecious trait. For gynoecious trait, it could precisely explain for around 80 %. However, it could not identify well for the subgynoecious phenotype. The subgynoecious phenotype was less stable in the field since the plant possesses only one dominant

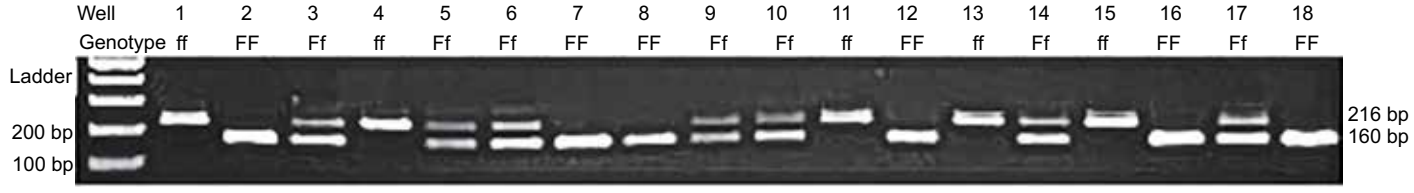

Fig. 1. 56 bp-deletion in *BCAT* 3'UTR could distinguish between homozygous and heterozygous F locus. DNA samples were isolated from gynoecious, subgynoecious, and monoecious cucumber leaves. PCR reactions were performed with a primer pair specifically binding to sequences around the 56 bp-deletion region in *BCAT* 3'UTR. PCR products were at 160 bp and 216 bp corresponding to F and f alleles, respectively.

Table 3. The correlation between marker genotype and sexual phenotype of plants in the three surveyed populations

Population	Genotype	Total number of plants	Phenotype			Marker genotype correctly matched with sex phenotype (%)	Overall (%)
			Gyoecious	Sub gynoecious	Monoecious		
Pure line	FF	10	4	-	6	40	54
	ff	3	-	-	3	100	
F2	FF	38	31	7	-	82	77
	Ff	49	-	26	23	53	
	ff	44	-	0	44	100	
F3.1	FF	7	7	-	-	100	69
	Ff	5	-	1	4	20	
	ff	1	-	-	1	100	
F3.2	FF	5	4	1	-	80	53
	Ff	8	1	2	5	25	
	ff	2	-	-	2	100	
F3.3	FF	0	-	-	-	-	100
	Ff	0	-	-	-	-	
	ff	15	-	-	15	100	
F3.4	FF	2	1	-	1	50	66.7
	Ff	4	-	-	4	0	
	ff	9	-	-	9	100	
F3.5	FF	1	1	-	-	100	43
	Ff	9		1	8	11	
	ff	4	-	-	4	100	

F allele and could be the possible justification for this. The data in this project is in consistent with previous report. Win *et al.* (2015) tested 55 inbred lines with different sexual phenotypes from different origins. They also found that the marker could perfectly distinguish monoecious trait and were least accurate for subgynoecious phenotype.

The marker sufficiently explained for Vietnam cucumber sex traits. Therefore, it could be applied for marker-assisted selection in cucumber breeding program in Vietnam.

BCAT- based marker is co-dominant marker that could distinguish between homozygous and heterozygous sex genotypes. Our group is running a cucumber improvement programme in Vietnam in which gynoecious trait is incorporated with disease resistance and other unique characteristics by backcrossing. *BCAT*-based marker is applied for gynoecious selection in each backcrossing.

Acknowledgements

We thank Truong Vu Quoc, Tan Loc Phat seed limited compary, for his kind support with cucumber cultivation. We acknowledge Ho Chi Minh City Open University for funding this study (E2017.8.016.2).

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Received: February, 2020; Revised: April, 2020; Accepted: April, 2020