การค้นหาส่วนของดีเอ็นเอที่แสดงความสัมพันธ์กับลักษณะกลายต้านทานโรคเส้นใบเหลืองในกระเจี๊ยบเขียวที่ได้จากการฉายรังสี

กนกพร บุญศิริชัย[*,1] วไลลักษณ์ แพทย์วิบูลย์[1] อำนวย อรรถลังรอง[2] วันเพ็ญ ศรีทองชัย[3] วิชัย ภูริปัญญวานิช[1]

[1]กลุ่มวิจัยและพัฒนานิวเคลียร์ สถาบันเทคโนโลยีนิวเคลียร์แห่งชาติ

โทรศัพท์ 0-2562-0114 โทรสาร 0-2561-4075 email: kanokporn@oaep.go.th

[2]สถาบันวิจัยพืชสวน กรมวิชาการเกษตร [3]สำนักวิจัยพัฒนาการอารักขาพืช กรมวิชาการเกษตร

บทคัดย่อ

ผสมพันธุ์กระเจี๊ยบเขียวพันธุ์กลายต้านทานโรคเส้นใบเหลืองกับพันธุ์พิจิตร 03 ซึ่งเป็นพันธุ์อ่อนแอ พบว่าลูกผสมสามารถต้านทานโรคได้ในระยะหนึ่งแต่จะแสดงอาการของโรคในที่สุด ทำการเปรียบเทียบลายพิมพ์ดีเอ็นเอระหว่างลูกผสม F_2 และ BC_1F_1 ที่ต้านทานโรคกับที่ไม่ต้านทาน พบว่าแถบดีเอ็นเอส่วนใหญ่ไม่แสดงความสัมพันธ์กับลักษณะใดลักษณะหนึ่ง แต่การเปรียบเทียบลายพิมพ์ดีเอ็นเอระหว่างพันธุ์กลายกับพันธุ์เดิมสามารถพบแถบดีเอ็นเอที่ต่างกันได้

คำสำคัญ : กระเจี๊ยบเขียว โรคเส้นใบเหลือง ลายพิมพ์ดีเอ็นเอ รังสีแกมมา

Identification of DNA Fragments that Showed Linkage to the Radiation-induced Yellow Vein Mosaic Disease Resistance Mutation in Okra

Kanokporn Boonsirichai[*,1] Valailak Phadvibulya[1] Amnuai Adthalungrong[2] Wanphen Srithongchai[3] and Vichai Puripunyavanich[1]

[1]Research and Development Division, Thailand Institute of Nuclear Technology, Tel.0-2562-0114, Fax.0-2561-4075 email: kanokporn@oaep.go.th [2]Horticultural Research Institute, Department of Agriculture

[3]Crop Protection Research and Development Office, Department of Agriculture

Abstract

The yellow vein mosaic disease resistant mutant of okra was crossed to Pichit 03, a susceptible variety. Their progeny showed prolonged resistance when compared with Pichit 03. DNA fingerprints of F_2 and BC_1F_1 individuals from the cross indicated that most DNA bands did not segregate with either the resistance or the susceptible characteristics. Nonetheless, polymorphic DNA bands could be identified between the mutant and Okura, the parental variety.

Keywords : okra, yellow vein mosaic disease, DNA fingerprint, gamma radiation

Introduction

Okra (*Abelmoschus esculentus*) is an economic vegetable crop of Thailand. Its export value totals over 323 million bahts in 2006. (The Customs Department, 2007). Major okra growing area is in the central region of the country, near Bangkok. Yellow vein mosaic disease (YVMD) has become widespread among okra populations since 1997, when it caused up to 50% reduction in okra productions. The symptoms include yellowing of leaf veins, yellow leaves and shoots, curling or rolling of shoots and top leaves and yellow fruits. Severely infected plants become stunted and show a reduction in fruit yield. YVMD is caused by a geminivirus which is transmitted by tobacco white fly (*Bemisia tabaci*).

As a solution to this problem, the Office of Atoms for Peace together with the Department of Agriculture has developed YVMD-resistant mutant okra lines, which were obtained through gamma radiation mutagenesis of the susceptible Okura variety. (Phadvibulya, *et al*., 2001). They exhibited prolonged resistance when compared with the parental variety and Pichit 03, another susceptible variety. A mutant line, B4610, which has good morphology and desirable fruit quality, was studied further. In this report, the dominance/recessiveness of YVMD resistance was determined and the DNA fingerprints of the mutant, the parental variety and segregating backcross progeny were studied in order to identify DNA fragments that are closely linked to YVMD resistance mutation.

Methods

1. Plant materials and growing conditions

B4610 YVMD-resistant mutant seeds belonged to the M9 and M10 generations. Pichit 03, wild-type Okura, and YVMD-resistant mutant were grown in the field at the Pichit Horticultural Research Center, Department of Agriculture, Pichit, where YVMD was widespread, for natural inoculation of YVMD.

Crosses between the mutant and Okura, or between the mutant and Pichit 03, were conducted and their progeny were planted in the field at the Pichit Horticultural Research Center, Pichit.

Greenhouse inoculations using white fly carrier were conducted on okra seedlings at the nursery belonging to the Crop Protection Research and Development Office, Department of Agriculture, Bangkok.

Susceptibility to YVMD was determined by visual inspection of the plants.

2. DNA extraction

DNA was extracted from frozen leaf tissues by a protocol modified from Dellaporta, *et al*. (1983). A fully-expanded leaf was ground to powder in liquid nitrogen. 15 ml extraction buffer (100 mM Tris-HCl, 500 mM NaCl, 50 mM EDTA, pH 8, 10 mM 2-mercaptoethanol) and 1 ml 20% SDS were added, and the mixture was incubated at 65^{o}C, 15 min. 5ml potassium acetate was added and the mixture was stored on ice for 10 min, followed by a 20-min centrifugation at 8,000 rpm., 15^{o}C. The supernatant was extracted with 0.75 volume of chloroform : isoamyl alcohol (24:1 vol/vol). DNA was precipitated from the aqueous layer by adding 0.5 volume of isopropanol followed by incubation at -20^{o}C for at least 30 min. The mixture was centrifuged at 10,000 rpm, 15^{o}C, 15 min, to collect the DNA. The DNA was resuspended in 800 μl 50 mM Tris-HCl, 10mM EDTA, pH 8, and it was re-precipitated by adding 80 μl 3M sodium acetate, pH 5.7, 550 μl isopropanol. DNA precipitates were collected by a 2-min centrifugation at 12,000 rpm., air-dried, resuspended in 10 mM Tris-HCl, 0.1 mM EDTA, pH 8, and stored at -20^{o}C until use.

3. DNA fingerprinting

AFLP fingerprinting was conducted as described by Vos *et al*., (1995) but using *Pst*I instead of *Eco*RI in most of the reactions. PCR reactions were done in two steps : pre-selective (with one selective nucleotide) and selective amplification (with three selective nucleotides). The *Pst*I adaptor sequences were 5'-CTC GTA GAC TGC CGT ACA TGC A-3' and 5'-TGT ACG CAG TCT AC-3' and the *Pst*I adaptor primer sequence was 5'-GAC TGC GTA CAT GCA GNN N-3', where Ns are selective nucleotides. MFLP fingerprinting was conducted as described by Yang, *et al*. (2001). The sequences of microsatellite primers are as in Yang, *et al*. (2001). The AFLP and MFLP products were separated on a 4.5% acrylamide, 7M urea gel Vos, *et al*., (1995) and stained with silver nitrate. (Promega Corporation, 2000).

Results and Discussion

1. Genetics of YVMD resistance

To determine the dominance/ recessiveness of YVMD resistance in B4610 mutant, the mutant was reciprocally crossed to Okura (the susceptible parental variety) and Pichit 03 (another susceptible variety). The resulting F1 seeds were planted in the field at the Pichit Horticultural Research Center, Pichit, where YVMD has been widespread. Progression of YVMD among the F1 and their parents is shown in Table 1. By 2 months, Okura and Pichit 03 started showing YVMD symptoms, which include yellowness of leaf veins and fruits (Fig. 1). At 2.5 months, all Okura individuals had already developed the symptoms, while the mutant and all of the F1 individuals showed no signs of the disease. However, after three months the F1 started to exhibit some signs of the disease. The data were confirmed by greenhouse inoculation of the F1 seedlings (Table 2). At 1.5 months, all of Pichit 03 plants had developed YVMD symptoms, while none of the F1 showed signs of the disease. Nonetheless, at 2.5 months many of the F1 started to show yellow coloration of leaf veins. Since the F1 individuals exhibited prolonged resistance compared to Okura and Pichit 03, it can be concluded that YVMD resistance behaved as a semi-dominant trait.

Table 1. YVMD susceptibility of naturally-inoculated okra populations.

Okra line	Number of plants germinated	Number of infected plants at various time after seeding				% infected plants at 2.5 months
		1 month	1.5 months	2 months	2.5 months	
B4610xOkura	32	0	0	0	0	0
OkuraxB4610	24	0	0	0	0	0
B4610xPichit03	22	0	0	0	0	0
Pichit03xB4610	37	0	0	0	0	0
B4610	44	0	0	0	0	0
Okura	23	0	0	3	23	100
Pichit 03	44	0	0	5	27	61

Fig. 1. Naturally-inoculated okra plants. A. B4610 x Okra. B. Okura x B4610. C. B4610 x Pichit03. D. Pichit03 x B4610. E. B4610, YVMD resistant mutant. F. Okura. G. Pichit03.

Table 2. YVMD susceptibility of greenhouse-inoculated okra populations.

Okra line	No. susceptible/total	
	1.5 months	2.5 months
Pichit 03	22/22	22/22
Okura x B4610	0/19	16/19
Pichit 03 x B4610	0/11	4/11

2. Analysis of DNA fingerprints of BC1F1 individuals

To identify DNA fragments that cosegregrate with YVMD resistance, MFLP fingerprints were performed on 26 YVMD resistant individuals and 26 susceptible individuals from the cross Pichit03 x (Pichit03 x B4610). 60 primer combinations, consisting of 6 microsatellite-anchor primers and 10 selective *Mse*I-adaptor primers, were analyzed. Most DNA fragments did not co-segregate with YVMD resistance. Only a single DNA fragment showed potential linkage to the resistance trait. It was obtained from selective amplification using MF43 microsatellite-anchor

primer and the *Mse*I-CAG selective primer (Fig. 2). The fragment exhibited a recombination frequency of 0.19 with YVMD resistance, and should therefore be located on the same chromosome as the mutation.

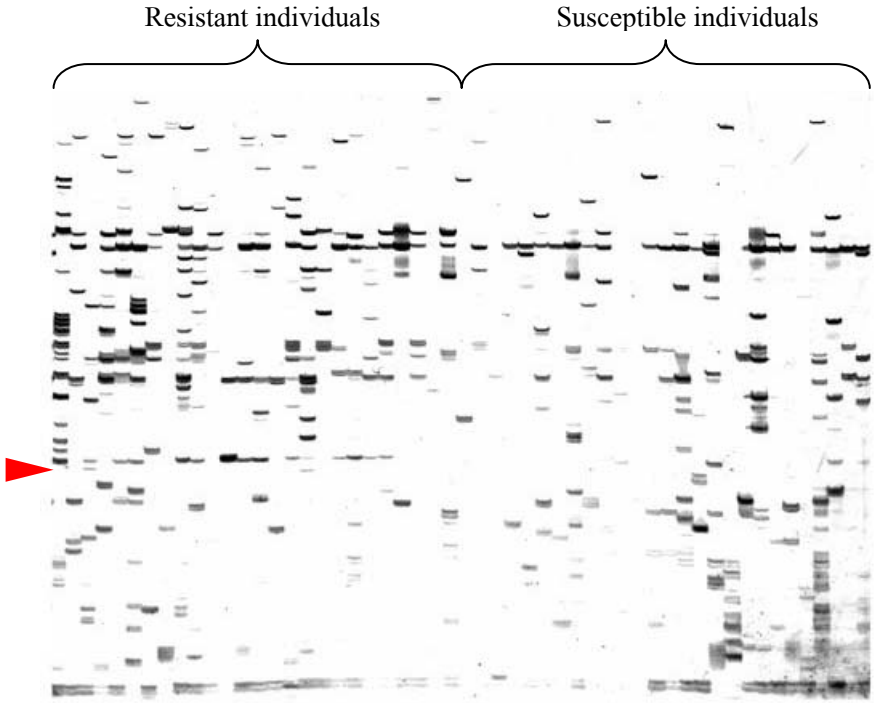

Fig. 2. MFLP fingerprints of BC1F1 individuals from the cross Pichit03 x (Pichit03 x B4610). The DNA fragment that is potentially linked to YVMD resistance is marked with an arrowhead.

3. Comparison of DNA fingerprints of the mutant and the parental variety

AFLP fingerprints of the mutant and the parental variety were compared using primers *Pst*I-ACT and *Mse*I-CCT. A DNA fragment was found to be present in the mutant fingerprints but absent from the parental fingerprints (Fig. 3). Although this DNA fragment might not be causative of YVMD resistance in the mutant, it might be useful for identification purposes when the mutant line is released to the public.

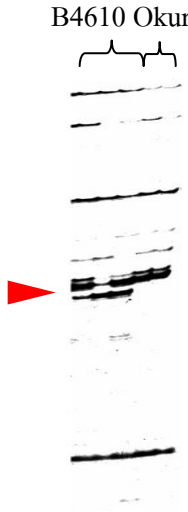

Fig. 3. AFLP fingerprints B4610, YVMD resistant mutant (3 individuals), and Okura (2 individuals) showing polymorphisms between the mutant and Okura (red arrows).

Conclusion

Analysis of the F1 progeny from crosses between B4610 mutant and susceptible varieties including Okura, its parental variety, and Pichit 03, suggested that YVMD resistance behaved as a semi-dominant trait. MFLP fingerprints of the BC1F1 individuals revealed a potentially linked DNA fragment exhibiting a recombination frequency of 0.19 with YVMD resistance. Thus, this DNA fragment should be located on the same chromosome as YVMD resistance mutation. However, additional fingerprints should be analyzed to identify fragments that showed fewer recombination events with the mutant trait. In the meantime, AFLP fingerprints also revealed a DNA fragment that was present in the mutant but lacking from the parental variety. This fragment might be useful for diagnostic purposes, when the YVMD-resistant line is released to the public.

Acknowledgements

This research is supported in part by the International Atomic Energy Agency research contract no. 12824. The authors wish to thank Miss Sirilak Chookaew and Miss Walayaporn Tongpun for technical support.

References

1. Dellaporta, S.L., Wood, J., Hicks, J.B., 1983. A plant DNA mini-preparation version II. Plant Mol. Biol. Reptr. 1, 19-21.
2. Phadvibulya, V., Puripanyavanich, V., Kittipakorn, K., Adthalungrong, A., 2001. Induce mutation for resistance to yellow vein disease in okra. in The 8th Nuclear Science and Technology Conference. Radiation and Life. Kasetsart University, Bangkok, Thailand. pp.53-62. (in Thai).
3. Promega Corporation, 2000. Silver Sequence™ DNA Sequencing System. Technical Manual No. 023. available at http://www.promega.com.
4. The Customs Department, 2007. Import/Export Statistics. http://www.customs.go.th/Statistic/StatisticIndex.jsp.
5. Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., Zabeau, M., 1995. AFLP: A new technique for DNA fingerprinting. Nucleic Acids Research 23, 4407-4414.
6. Yang, H., Sweetingham, W., Cowling, W.A., Smith, P.M.C., 2001. DNA fingerprinting based on microsatellite-anchored fragment length polymorphisms, and isolation of sequence-specific PCR markers in lupin (*Lupinus angustifolius* L.). Molecular Breeding 7, 203-209.