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การเติบโตโกงกางใบใหญ่ด้วยหัวเชื้อชีวภาพอัดเม็ด *Trichoderma* บริเวณดินตะกอนเลนที่ดักด้วยแนวไม้ไผ่ปากแม่น้ำท่าจีน.......81

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การคัดกรองเครื่องหมายโมเลกุลของยีนต้านทานโรคใบไหม้ในหน้าวัว

Screening for Bacterial Blight Resistance Molecular Marker of *Anthurium andreanum*

ณัฐพงษ์ ศรีสมุทร [1*] และ แก้วตา สูตรสุวรรณ [1]

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บทคัดย่อ

การวิจัยครั้งนี้มีวัตถุประสงค์เพื่อค้นหายีนหรือแถบดีเอ็นเอที่จำเพาะต่อลักษณะการต้านทานโรคใบไหม้ในหน้าวัว (*Anthurium andreanum* Lind.) โดยพิจารณาแถบดีเอ็นเอที่พบเฉพาะในหน้าวัวสายพันธุ์ที่มีความต้านทานต่อโรคสูงแต่ไม่พบในสายพันธุ์ที่เป็นโรค การจำแนกชนิดของเชื้อแบคทีเรียสาเหตุโรคใบไหม้ของหน้าวัว (*Xantomonas axonopodis* pv. *dieffenbachiae*; *Xad*) โดยศึกษาลักษณะทางสัณฐานวิทยา สรีรวิทยาและชีวเคมีของเชื้อแบคทีเรียที่แยกได้จำนวน 93 ตัวอย่าง ผลการทดลองพบแบคทีเรียที่มีลักษณะของ *Xad* จำนวน 12 ตัวอย่าง นำเชื้อบริสุทธิ์ที่แยกได้มาทำให้เกิดโรคในหน้าวัวสายพันธุ์ต่างๆ พบว่า หน้าวัวสายพันธุ์เปลวเทียนลำปางมีความต้านทานโรคสูงที่สุด สายพันธุ์ยาควีนต้ามีความต้านทานโรคต่ำที่สุด ที่ระดับความรุนแรงของโรคเฉลี่ยหลังจากปลูกเชื้อ เท่ากับ 0.40 และ 3.80 ตามลำดับ การสร้างลายพิมพ์ดีเอ็นเอโดยใช้ไพรเมอร์ไอเอสเอสอาร์ (ISSR primer) ด้วยปฏิกิริยาลูกโซ่พอลิเมอเรส พบว่ามีไพรเมอร์ที่สามารถให้แถบดีเอ็นเอกับหน้าวัว 20 สายพันธุ์ จำนวน 18 คู่ไพรเมอร์ เมื่อทำการตรวจหาแถบดีเอ็นเอที่ปรากฏเฉพาะในหน้าวัวสายพันธ์ที่ต้านทานโรค คือ สายพันธุ์เปลวเทียนลำปาง ผลการทดลองไม่พบแถบดีเอ็นเอที่มีปรากฏเฉพาะในหน้าวัวสายพันธุ์ดังกล่าวทั้งนี้อาจเนื่องมาจากลายพิมพ์ไอเอสเอสอาร์ให้แถบจำนวนมากจึงไม่เกิดความแตกต่างระหว่างสายพันธุ์ที่ต้านทานโรคกับสายพันธุ์ที่อ่อนแอจึงไม่สามารถระบุแถบดีเอ็นเอที่จำเพาะต่อลักษณะการต้านทานโรคใบไหม้ในการศึกษาครั้งนี้ได้ดังนั้นผู้วิจัยจะทำการศึกษาเพิ่มเติมต่อไป

คำสำคัญ: ยีน, โรคใบไหม้, หน้าวัว, ไอเอสเอสอาร์

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ABSTRACT

The objective of this study was to determine the specific characteristics of bacterial blight resistance gene of *Anthurium andreanum* Lind. by considering the DNA bands found only in the resistance cultivars, but not in the weak ones. According to the morphological, physiological, and biochemical classifications of 93 bacterial samples, there were 12 isolates share the similar characteristics as *Xanthomonas axonopodis* pv. *dieffenbachiae* (*Xad*), which is the cause of bacterial blight. The artificial inoculation test showed Plewtien Lampang cultivar contains the highest disease resistance with average violence level of 0.40; while the lowest disease resistant cultivar was La Quinta which showed the highest average violence level of 3.80. DNA fingerprints using ISSR primers indicated that 18 primer pairs could reveal DNA patterns of 20 *Anthurium* cultivars. However, there was no specific DNA band of the bacterial blight resistance found in Plewtien Lampang cultivar. This may be due to the large number of bands generated by the ISSR fingerprinting; the difference between the resistance and weak varieties was unable to distinguish. In short, the specific characteristics of bacterial blight resistance gene could not be identified in this study and the further research should be conducted.

Key words: bacterial blight, gene, *Anthurium*, ISSR

INTRODUCTION

Anthurium is the most speciose genus in the Araceae family, a monocot family defined by its unique inflorescence composed of a spadix and spathe. The spadix holds hundreds of mini flowers compacted on a spike, which is subtended by a more or less showy sterile leaf-like organ, the spathe (Bliss and Suzuki, 2012). *Anthurium* was reported to encompass more than 1,500 species. Numerous *Anthurium* species were produced and traded internationally as cut-flowers (*Anthurium andreanum* Linden.), flowering potted plants and landscape plants (Nowbuth *et al.*, 2005). The novel colors and forms, as well as desirable horticultural attributes, generated in these cultivars contributed to the dominance of the *Anthurium* industry. However, the major problem of *Anthurium* production is diseases including black rot, root rot, anthracnose, and bacterial blight.

Bacterial blight caused by *Xanthomonas axonopodis* pv. *dieffenbachiae* (*Xad*). Bacterial blight is one of the major diseases of *Anthurium*. It was firstly reported from Brazil in 1960 and prevalent in almost *Anthurium* growing regions all over the world (Dhanya and Mary, 2006). In order to control the disease outbreak, antibiotics such as streptomycin sulphate and

oxytetracycline are frequently recommended. However, excessive use of these antibiotics may contaminate in the environment (Dhanya and Mary, 2006). There are several attempts to develop new cultivars with high resistance to disease but the development of new cultivars with high resistance to disease mainly relies on the genetic information. Thus, understanding the specific characteristics of bacterial blight resistance gene is the prerequisite to the development of molecular resources in order to support the improvement of *Anthurium* cultivars. In addition, the specific molecular markers can establish the identity of the registration material and cultivars protection.

The biotechnology has developed rapidly over the years, and the molecular markers have become important tools in the genetic research and assist in breeding efficiency. Among then, the Inter-simple sequence repeats (ISSRs) technique has provided a powerful, rapid, simple, reproducible and inexpensive means to assess the genetic diversity and closely identify related cultivars in many species. The ISSR technique has been extensively used for several applications in molecular taxonomy, conservation, and breeding (Kumar *et al.*, 2009). At present, ISSR has been successfully applied to detect the genetic similarities or dissimilarities in various plants (Carvalho *et al.*, 2004), such as drought sensitive and drought tolerant genotypes of wheat (*Triticum aestivum* L.) (Barakat *et al.* 2013), genetic variation among the radiated population of banana (Khatri *et al.*, 2011), and genetic diversity analysis of African edible-seeded cucurbits (*Citrullus lanatus* L., *Cucumeropsis mannii* L. and *Cucumis melo*) (Djè *et al.*, 2006). Furthermore, ISSR is useful in fingerprint and characterization of accessions and identification of verities containing gene that produces a specific trait. ISSR technique involves the use of a microsatellite core unit bearing oligonucleotide primers, usually 16–25 bp long, nonanchored or anchored at the 5' or 3' end with 1–4 degenerate nucleotides. ISSR can be a rapid and easy technique for identifying specific trait gene. This technique overcomes many technical limitations of RAPD and AFLP because of its high reproducibility and simplicity (Bornet and Branchard, 2001). Due to these benefits of ISSR marker, the aim of the present study was to find the specific characteristics of bacterial blight resistance gene of *Anthurium* by considering the DNA band found only in the cultivars with resistance to the bacterial blight but not in the weak cultivars.

MATERIALS AND METHODS

Sample collection and disease diagnosis

The *Xad* was isolated from symptomatic *Anthurium* leaf tissue collected from Division of Biotechnology, Kalasin University, Thailand during the summer of 2012. To perform isolations, water-soaked tissue from the margin of typical lesions was rinsed under running chlorinated tap water. About 2 g of each sample was chopped into small pieces and placed into 15 mL falcon tubes containing 3 mL of sterile

water. The samples were incubated at room temperature for 30 minutes to allow the bacteria to ooze into water. One hundred microliters of the suspension was streaked on plates of nutrient agar (NA) and incubated at 28°C for 96 h. Yellow mucoid colonies, characteristic of *Xad*, were further sub-cultured into fresh media until pure colonies were obtained. These colonies were confirmed as *Xad* by performing a pathogenicity test (Schaad *et al.*, 2001).

Pathogenicity testing

Xad isolated from infected *Anthurium* were tested for pathogenicity by Koch's postulates. One hundred microliters (equal to 1 x 10^8 CFU mL^{-1}) were sprayed onto the *Anthurium* leaf until runoff occurred. Three plants were inoculated with *Xad* and one plant was inoculated with sterile water as control. The plants were incubated inside plastic bag in a growth chamber and kept a constant 28°C for 3 weeks. The incidence of symptoms was recorded weekly starting at 3 days after inoculation, and the identity of the pathogen was confirmed by re-isolation from arbitrarily selected plants as described previously.

Evaluation of resistance to bacterial blight in *Anthurium* cultivars

Twenty *Anthurium* cultivars were screened for resistance to bacterial blight (Table 1). Plants were grown in plastic containers in the greenhouse for 6 weeks. Each cultivar contained of 5 replicate was inoculated with *Xad* and one was inoculated with sterile water as control. The leaves of all plants were sprayed with inoculum prepared as described previously. Inoculated plants were maintained in the greenhouse and enclosed with a sheet of plastic overnight immediately after inoculation to increase humidity and promote infection. Disease severity ratings were taken at 6 weeks postinoculation using the 0 to 5 (Little and Hills, 1978) as follows: 0 = no symptoms; 1 = ≥1%-≤10%; 2 = ≥10%-≤ 35%; 3 = ≥35%-≤65%; 4 = ≥65%-≤90% and 5 = ≥90% -≤100%.

***Anthurium* DNA extraction and ISSR analysis**

About three grams of leaf tissue were ground into a fine powder using liquid nitrogen in a mortar. Total genomic DNA was extracted with modified protocol of Gawel and Jarret (1991). DNA quality was evaluated on a 0.8% agarose gel stained with ethidium bromide. DNA Quantity was measured using a spectrophotometer and the DNA samples were diluted to 60 ng μL^{-1} for polymerase chain reaction (PCR) amplification. For PCR amplification, 18ISSR primers with a few selective nucleotides as anchors into the non-repeat adjacent regions (16-18bp) (Khatri *et al.*, 2011) were used (Table 2). These were carried out in a final volume of 25 µL, containing 60 ng of DNA, 0.5 U *Taq* polymerase, 2.5 µL of 10X reaction buffer, 3.0 mM $MgCl_2$, 0.2 mM of each dNTP and 0.5 µM primer. DNA amplifications were performed in the thermo cycler (TGradient 96, Biometra),

Table 1 Bacterial blight severity from artificial inoculation.

No.	Cultivar	Disease severity ratings						Avg. disease rating	Resistance category
		R1	R2	R3	R4	R5	Cont.		
1	Arizona	3	3	4	3	2	0	3.00	MS
2	Cherry Pink	2	2	2	2	2	0	2.00	MS
3	ChompooNopon	2	1	1	1	2	0	1.40	MS
4	Choke Goa Chan	1	1	1	1	2	0	1.20	MS
5	Dakota	2	2	1	1	2	0	1.60	MS
6	Gemini	2	1	1	1	2	0	1.40	MS
7	Janny Red	3	2	3	3	3	0	2.80	MS
8	JoaSuo	3	3	3	2	2	0	2.60	MS
9	La Quinta	4	4	3	4	2	0	3.40	HS
10	Meringue	2	2	2	2	2	0	2.00	MS
11	Pink Champion	1	2	2	2	1	0	1.60	MS
12	Pistache	2	2	1	1	2	0	1.60	MS
13	PlewtienLampang	1	0	0	0	1	0	0.40	R
14	Plewtien Phuket	1	1	1	0	0	0	0.60	R
15	Red Bar	3	3	3	3	3	0	3.00	MS
16	Red Strong	2	1	1	1	2	0	1.40	MS
17	Sang Tien	2	2	3	2	2	0	2.20	MS
18	Smission	1	1	2	2	1	0	1.40	MS
19	Sun Red	2	2	3	3	2	0	2.40	MS
20	Ta Ole	2	1	3	2	2	0	2.00	MS

under the following conditions: preliminary step of 10 min at 94°C, followed by 40 cycles of 40 sec denaturation at 90°C, 45 sec to annealing temperature by primer (Table 2) and 90 sec extension at 72°C with a final 7 min extension at 72°C. PCR products were electrophoretically resolved on 1.5% agarose gels run at 110 V in TBE 1X buffer and visualized by staining with ethidiumbromide (0.05 mg/mL). The obtained bands were compared with the 1kb DNA ladder marker (Invitrogen). In order to evaluate the reproducibility of the DNA profile, PCR reactions were carried out 3 times, and only well-defined and reproducible bands were considered. The DNA band which found only in the resistant cultivar was assumed as the bacterial blight resistance gene.

RESULTS AND DISCUSSION

Confirmation of pathogenicity

From 93 isolates of bacteria, 12 of these were *Xad* (data not show). All yellow mucoid

Table 2 List of primers and the number of amplified products detected.

Primer Name	Sequence (5'→3')	Total no. of bands	No. of polymorphic bands	% of polymorphic bands
P1	AGAGAGAGAGAGAGAGG	13	11	84.62
P2	CTCTCTCTCTCTCTCTAC	9	9	100.00
P3	CTCTCTCTCTCTCTCTGC	15	15	100.00
P4	CACACACACACAAC	8	6	75.00
P5	CACACACACACAGT	17	17	100.00
P6	CACACACACACAAG	13	12	92.31
P7	CACACACACACAGG	13	13	100.00
P10	GAGAGAGAGAGACC	9	8	88.89
P11	GTGTGTGTGTGTCC	12	12	100.00
P12	CACCACCACGC	15	15	100.00
P13	GAGGAGGAGGC	15	13	86.67
P14	CTCCTCCTCGC	17	17	100.00
P15	GTGGTGGTGGC	9	8	88.89
P16	ACTGACTGACTGACTG	13	11	84.62
P17	GACAGACAGACAGACA	15	15	100.00
P19	ACACACACACACACACG	9	9	100.00
P22	CCCCGTGTGTGTGTGT	13	13	100.00
P23	AGAGAGAGAGAGAGAG	8	8	100
	Total	223	212	95.07

colonies were pathogenic to *Anthurium* and the symptoms were firstly visible at 1 week after inoculation. Three weeks after inoculation, at least two-thirds of plants were show the signs of *Xad*. Negative control plants (non-inoculated) also remained healthy. The pathogen was recovered from symptomatic plants will be used for measured the resistance levels of bacterial blight in 20 *Anthurium* cultivars.

Many strains of pathogen should be tested from multiple locations in order to classified *Anthurium* cultivars with broadly resistance to *Xad*. The pathovar (pv.), *dieffenbachiae*, is very heterogeneous. It was subdivided into two biotypes distinguished by their ability to hydrolyze starch (Norman *et al*., 1999). Lipp *et al.* (1992) found 62% of 177 isolates tested were unable to hydrolyze starch in *Anthurium* cut-flower production in Hawaii. This pathovar diversity has been further suggested in studies of fatty acid profiles, metabolic profiles, and monoclonal antibodies (Norman *et al*., 1999). The starch-hydrolyzing biotype appears to produce symptoms more rapidly than the non-starch-hydrolyzing biotype (Norman *et al*., 1999).

Evaluation of resistance to bacterial blight

The disease severity was observed among cultivars of 6 weeks postinoculation. Disease severity ratings were collected rank from 0.40 in Plewtien Lampang cultivar to 3.80 in La Quinta cultivar. Moreover, Plewtien Phuket cultivar also shows high level of disease resistance with low severity ratings at 0.60. The average disease severity rating was used to classify cultivars based on susceptibility to bacterial blight (Seijo *et al.*, 2010). Cultivars with an average rating less than 1 were classified as resistant (R), 1 to less than 3 as moderately susceptible (MS), and 3 or higher as highly susceptible (HS) (Table 1). All cultivars were classified as R including Plewtien Lampang and Plewtien Phuket cultivars which significantly less disease than those classified as MS and HS (La Quinta). Although there is no existing information regarding the action mechanism of bacterial blight resistance in *Anthurium* but the similar responses of closely related cultivars were reported by Norman *et al.* (1999) which indicated a probable genetic basis for resistance.

There was a wide range of susceptibility to *Xad* among *Anthurium* cultivars. Only two cultivars, Plewtien Lampang and Plewtien Phuket, were identified as R cultivars while the mainly were MS. Previous studies (Norman and Alvarez, 1994), it was clear that cultivars with some level of resistance could have quiescent infections and carry the pathogen without showing symptoms. Thus, care should be taken to avoid planting infected R plants adjacent to HS cultivars (Seijo *et al.*, 2010). Available R cultivars may not suitable for all applications; therefore, the new improved cultivars are necessary to develop in order to provide R cultivars for being resistant from various disease problems occur in *Anthurium*. The bacterial strains with virulence to R cultivars will be selected over time; thus, the continuous cultivar improvement is highly required.

ISSR analysis

The PCR amplification using ISSR primers gave rise to reproducible amplification products (Figure 1). ISSR primers produced different numbers of DNA fragments, depending upon their simple sequence repeat motifs. According to the manual counting of DNA band, the 18 primers produced 223 bands across 20 *Anthurium* cultivars. These 212 bands were polymorphic, accounting for 95.07% polymorphism. The results obtained for each primer are presented in Table 2. The ISSR amplification generated an average of 13.12 DNA bands per primer. The size of the scorable amplified fragments ranged from 300 to 1500 bp. Primer P5 and P14 generated a high number of markers (17) while the lowest (8) was generated by primer P4 and P23.

DNA specific band for bacterial blight resistance

According to the R cultivars, Plewtien Lampang and Plewtien Phuket, they might contain genetic background for bacterial blight

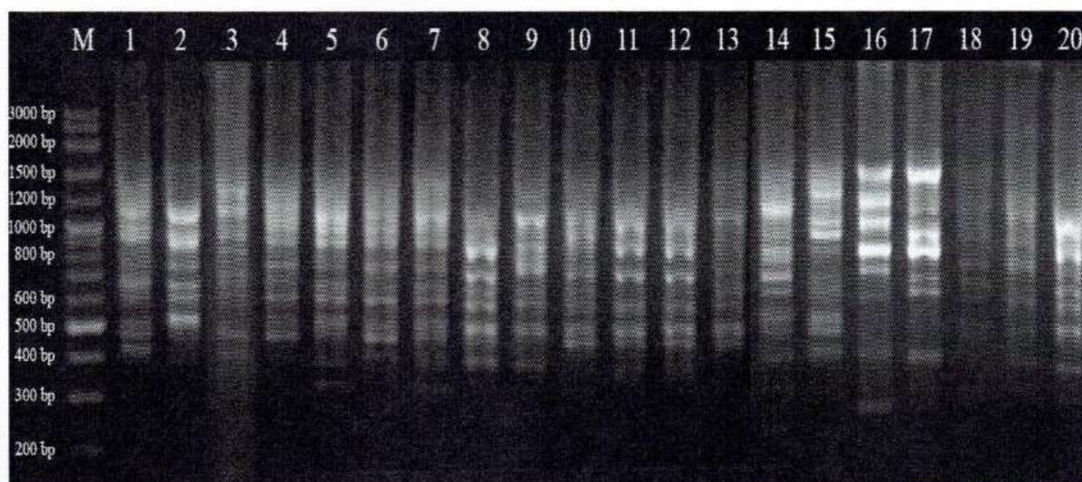

Figure 1 ISSR amplification pattern of 20 *Anthurium* cultivars obtained with primer P12. M is the DNA marker, lanes 1-20 are refer to number of *Anthurium* cultivars listed in Table 1.

resistance phenotype. Then, the DNA band that found only in these cultivars and not found in MS and HS cultivars could be referred to the bacterial blight resistance gene. However, the DNA band that appears only in Plewtien Lampang and Plewtien Phuket cultivars was not found in this study. This may be probably affected by large number of DNA bands generated from ISSR technique. The specific DNA band for bacterial blight resistance gene was subsequently unable to identify in this study. Moreover, the primers used and/or the separated method of DNA fragment might not be appropriate. This limitation could be solved by separating on polyacrylamide gel electrophoresis. On the other hand, these results could be useful in planning breeding programs in order to produce *Anthurium* hybrids resistant to *Xad* with the condition that the specific DNA band was found.

CONCLUSION

Twelve yellow mucoid colonies were *Xantomonas axonopodis* pv. *dieffenbachiae*. Bacterial blight symptoms were found at the first week after inoculation. Sprayed leaves were show signs of *Xad* at three week postinoculation while non-inoculated leaf also remained healthy. Disease severity ratings were lowest in Plewtien Lampang cultivar and highest in La Quinta cultivar which the average disease rating was 0.40 and 3.80, respectively. 223 DNA bands were generated across 20 *Anthurium* cultivars by using 18 ISSR primers; 212 of these bands were polymorphic. Plewtien Lampang and Plewtien Phuket cultivars were classified as R cultivar. However, the DNA band appeared only in R cultivar was not found in this study.

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