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MARKER ASSISTED SELECTION FOR RESISTANCE TO NORTHERN CORN LEAF BLIGHT IN SWEET CORN

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SUMMARY

Marker assisted selection for resistance to Northern Corn Leaf Blight (NCLB), caused by *Exserohilum turcicum*, has been carried out for 3 years at Maejo University, Thailand. The objectives were to: (1) screen resistant (R) and susceptible (S) sweet corn inbreds for *E. turcicum*, (2) identify polymorphic SSR markers closely-linked to the known *Ht* genes for NCLB resistance, and use these polymorphic markers for selection of F_2 progenies. One highly S line (NT58WS_6#4) and 3 highly R lines (ChallengerS_6-1, Sugar73S_7-18 and hA4135) were identified and used for population development. Three SSR primers (umc1042, bnlg1721 and umc1149) showed polymorphism between susceptible and resistant lines. The 157 F_2 plants were used to determine linkage between trait and markers. Only 2 SSR primers about 6.7 cM on chromosome 2; bnlg1721 and umc1042 were closely-linked to the resistant gene *Ht1* (R^2 = 0.2948 and 0.2626, respectively, $P < 0.0001$). These 2 SSR primers may be useful as molecular markers for NCLB resistance in sweet corn.

Key words: Sweet corn, northern corn leaf blight, resistance, SSR marker, assisted selection

Key findings: 2 SSR markers, umc1042 and bnlg1721 were closely linked to the resistant gene (*Ht1*). These markers might be used as indicator for NCLB resistance in sweet corn.

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INTRODUCTION

Sweet corn is grown as a commodity crop in Thailand for fresh and frozen consumption as well as the canned industry for export. In 2013, 167,011 metric tons of canned sweet corn were exported with a value of $168.75 million. In the past decades, Northern Corn Leaf Blight (NCLB) has been a major foliar disease of corn (*Zea mays* L.) causing substantial yield losses in mid-altitude and highland regions of the tropics and subtropics (Leonard *et al.*, 1989); (Lothrop, 1989). It is caused by the fungus *Exserohilum turcicum* (Pass.) and was previously called *Helminthosporium turcicum* (Pass.) (Leonard *et al.*, 1989). Symptoms of NCLB are elliptical, chlorotic leaf lesions which start as gray-green color and later become necrotic and wilted. Lesions may coalesce in susceptible plants and complete destruction of the foliage. NCLB has been reported in Thailand since 1964, but it is not so severe because there is no widespread

cultivation of sweet corn. Since 2004, sweet corn production has been widespread for use in the food industry, with extensive plantings in the rainy and late rainy seasons in irrigated areas. The favorable environment conditions for the disease are at relatively low temperatures, especially 18-27 °C at night and about 90-100% relative humidity which is conducive to the growth of fungus (Juliana *et al*., 2005).

The most effective way to control NCLB is to use resistant cultivars. Most corn breeders prefer the use of quantitative NCLB resistance in their cultivar development programs (Welz and Geiger, 2000). Several qualitative NCLB resistance genes (*Ht*) have been identified, namely *Ht1, Ht2, Ht3* and *HtN* referring to their locus designations (Hooker, 1963, 1975, 1977); (Hooker and Kim, 1973); (Gevers, 1975). According to Wang *et al*. (2012), the resistant gene controlling NCLB disease were either dominant or quantitative gene actions. The resistance gene *HtN* is located on chromosome 8, flanked by MAC216826-4 and umc2218 at distance of 3.3 and 3.4 cM, respectively. Among Thai sweet corn germplasm, there is limited information on *Ht* genes and available markers to apply marker assisted selection (MAS) in breeding for NCLB resistance sweet corn. Thus the following objectives were developed in order to identify resistant (R) and susceptible (S) sweet corn lines by inoculation with *E. turcicum* and developing F_1 populations between R and S lines, as well as to identify polymorphic SSR primers between the R and S lines and to find associated markers to resistant genes and then apply marker assisted selection for NCLB resistance in sweet corn lines.

MATERIALS AND METHODS

Plant material

Four sweet corn inbred lines were inoculated with *E. turcicum* to determine their resistance to NCLB, with rating score from 1 (resistant) to 9 (susceptible). Three of the lines, namely ChallengerS_6-1 (P2), Sugar73S_7-18 (P3) and hA4135 (P4) were scored 1 as resistance. In contrast, NT58WS_6#4 (P1) was scored 9 as susceptible. These R and S lines were used as male and female parents to develop F_1 populations; Population 1 (P1 x P2); NT58WS_6#4 x ChallengerS_6-1, Population 2 (P1 x P3); NT58WS_6#4 x Sugar73S_7-18 and Population 3 (P1 x P4); NT58WS_6#4 x hA4135. The three F_1 populations were further studied for DNA polymorphism between the parents and F_1 by polymerase chain reaction (PCR) using SSR markers. Only population 1 which was derived from an elite hybrid gaining high consumption quality was self-pollinated to make F_2 populations as marker assisted selection to resist NCLB in sweet corn.

Inoculation and disease evaluation

The disease inoculum was originally isolated from corn grown in Chiang Mai, Thailand. The inoculation and disease evaluation were conducted in 2011 and 2013, late rainy season. Spore suspensions of *E. turcicum* at a concentration of 10^5 conidia per ml were inoculated into mid leaves at two-leaf stage (V2) at 14 days after planting. Severity of NCLB disease was assessed by scoring 1-9 at 56 days after planting, based on the percentage of the total leaf area affected using a slightly modified version of the standard diagram (Elliot and Jenkins, 1946).

Marker analysis

SSR markers linked to or closely located to the known *Ht* genes; *Ht1*, *Ht2*, and *HtN1* were identified from the Maize Genetics and Genomics Database (2012). Thirty-two SSR primers were used to amplify DNA from R and S parents to identify which SSRs are polymorphic and can be used for further studies (Table 1) The genomic DNA from the parents and F_2 individuals was extracted using a genomic DNA purification kit (obtained from Fermantas Company). A 15 µl PCR reaction contained dH_20 1.5 µl, 2x Promega's PCR green master mix (400 µM dNTPs reaction buffer pH 9, 3 mM $MgCl_2$ and Taq DNA polymerase 0.1 units/µl) 7.5 µl, SSR primers solution forward 2 µl, SSR primers solution reverse 2 µl and DNA template 2 µl. Amplification of DNA templates by PCR reaction included, predenaturing at

94 °C for 2 minutes, followed by 35 cycles of PCR amplification using the following parameters: denaturing at 94 °C for 1 minute, annealing at 53 °C for 2 minutes, extension at 72 °C for 2 minutes and final extension at 72 °C for 5 minutes. PCR products were size fractionated in 4% agarose gel with 1x TBE buffer at 80 V for 3 hours and then photographed by Gel documentation. The size of PCR products were determining by comparison with 100 bp DNA ladder size standard. The 157 F_2 DNAs from population 1 relevant polymorphic primers were associated with resistant genes (Asea *et al.,* 2009). The marker score was calculated by summing across marker loci the product of the coded marker genotype (0, 1 and 2) as described for marker-based selections (Lande and Thompson 1990; Moreau *et al.,* 2000). A value of 0 was given to a homozygous resistant loci, 1 for heterozygous and 2 for homozygous susceptible loci. Associations between individual marker loci and disease severity were tested with single-factor analysis of variance using the SAS version 8.0.

Table 1. SSR primers closely linked to NCLB resistance gene.

No. of Primers	Gene	Chromosome	Gene Position
12	*Ht1*	2	206,643,048 to 207,194,203
10	*Ht2*	8	135,858,136 to 398,042,717
10	*HtN1*	8	161,901,417 to 162,533,647

RESULTS

The screening of resistance to NCLB of sweet corn inbred lines was carried out in 2011 late rainy season. Three lines were identified highly resistant: ChallengerS_6-1, Sugar73S_7-18 and hA4135 with disease score of 1. In contrast, NT58WS_6#4 was scored as 9 and classified as highly susceptible.

The development of F_1 sweet corn populations from S_6 inbred lines was generated by using the highly susceptible (Score = 9) as a female dissimilar to the highly resistant male line (Score = 1). As a result, three F_1 populations were derived: They would be further used for detecting polymorphism between resistant and susceptible genotypes by SSR primers.

To determine polymorphism of the 32 SSR markers identified as co-located with known *Ht* genes; *Ht1*, *Ht2* and *HtN1*, PCR analyses of DNA from the R and S inbreds and three F_1 populations derived from them were carried out. Figure 1 shows polymorphism of umc1042 across R and S lines as well as the pooled F_1s of Population 1 (P1 x P2), Population 2 (P1 x P3), Population 3 (P1 x P4). It shows that the susceptible line NT58WS_6#4 (Lane1) has a different fragment size amplified by umc1042 compared to the R lines ChallengerS_6-1 (Lane 2), Sugar73S_7-18 (Lane 6) and hA4135 (Lane 10) wherein 2 fragments were amplified. Population 1 (Lane 1, 2, 3 and 4) showed similar patterns of the DNA amplification with Population 2 (Lane 5, 6, 7 and 8). Similar results were obtained for bnlg1721 and umc1149 primers (Figures 2 and 3). Based on these results, umc1042, bnlg1721 and umc1149 can differentiate between R and S lines which co-located with *Ht1* (umc1042 and bnlg1721) and *HtN1* (umc1149). Therefore, these 3 primers were used in subsequent studies as potentially linked to known *Ht* genes; *Ht1* (umc1042 and bnlg1721) in chromosome 2 and the *HtN1* (umc1149) in chromosome 8 (Table 2).

Table 2. List of relevant SSR primers showed polymorphic patterns in susceptible and resistant sweet corn lines, amplified within the three F_1 populations; Population 1, Population 2 and Population 3.

ID	Chr.	Gene	Forward primer	Reverse primer
umc1042	2	*Ht1*	AAGGCACTGCTACTCCTATGGCTA	CTGACCTTTGAATTCTGTGCTCCT
bnlg1721	2	*Ht1*	ACGACTTTCATGCCTCGTCT	ATTTCTTTTGCCACCTCAGC
umc1149	8	*HtN1*	TACAGTAGGGATTCTTGCAGCCTC	GTGGGACCTTGTTGCTTCCTTT

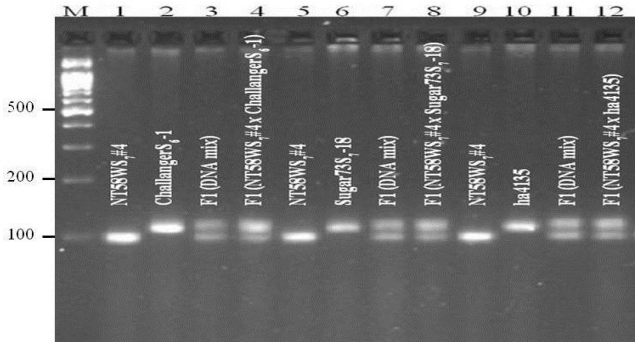

Figure 1. Gel electrophoresis showing presence polymorphic patterns between susceptible, resistant parents and F_1 were generated by using umc1042.

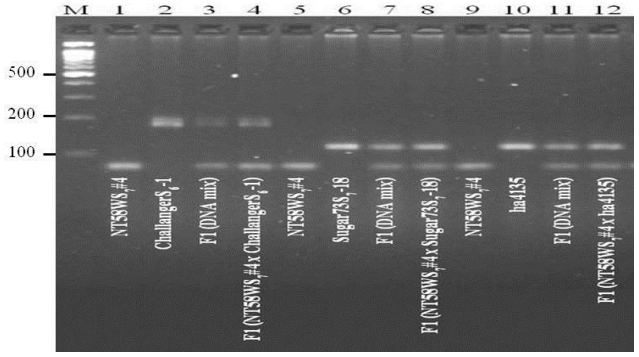

Figure 2. Gel electrophoresis showing presence polymorphic patterns between susceptible, resistant parents and F_1 were generated by using bnlg1721.

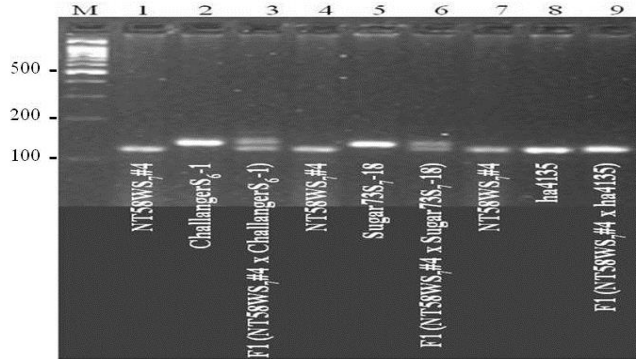

Figure 3. Gel electrophoresis showing presence polymorphic patterns between susceptible, resistance parents and F_1 in Population 1 and Population 2, were generated by using umc1149.

The 157 F_2 segregating individual plants of Population 1 were grown in order to validate the relevance of markers; umc1042, bnlg1721 and umc1149 for marker-assisted selection. Figures 4 and 5 ; the P1, P2, F_1 and 1-80 (Y), 81-160 (W) F_2 DNAs were PCR amplified with umc1042 polymorphic primer and size fractionated in 4% agarose gel. The individual F_2 DNAs distributed similarly to homozygous susceptible parents (P1), homozygous resistant parents (P2) and heterozygous (P1 x P2).

Similar results were also obtained using bnlg1721 and umc1149 primers. The three distinct genotypic classes were tested by Chi-square analysis. Results show that the Chi-square values obtained, 0.27, 0.82 and 0.26 respectively, were less than χ^2 ($P \leq 0.05$) of 5.99 or non-significant. Therefore, all the genotypes in F_2 fit in the expected 1:2:1 ratio (Table 3).

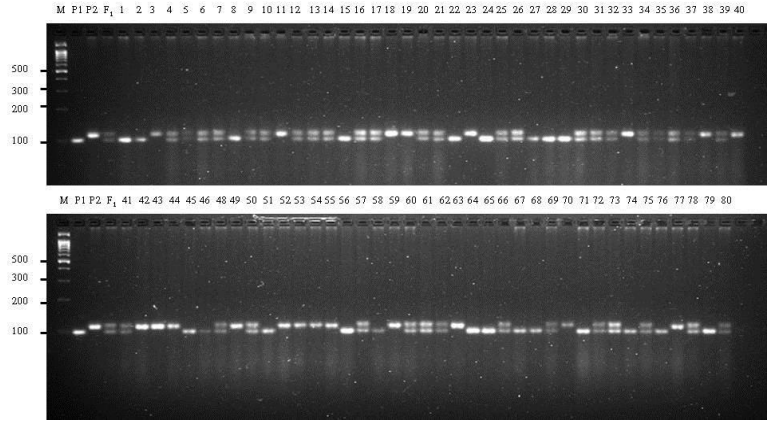

Figure 4. DNA amplification by umc1042 on P1(susceptible parent NT58WS$_7$#4) , P2 (resistant parent ChallengerS$_6$-1), F_1 and 1-80 F_2 (Y) individual plants of Population 1; (NT58WS$_6$#4 x ChallengerS$_6$-1).

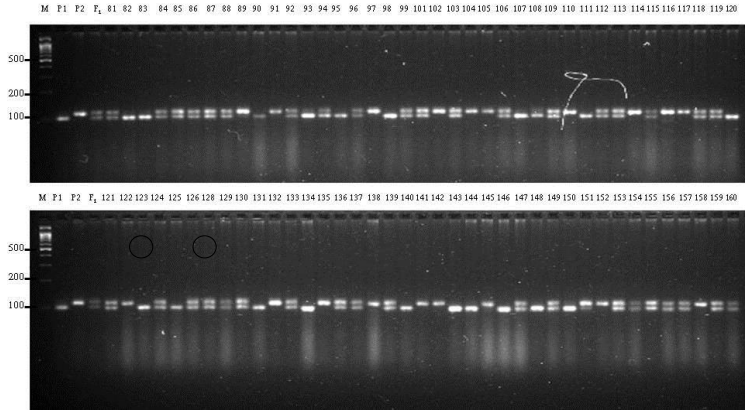

Figure 5. Gel electrophoresis amplified by umc1042 on P1, P2, F_1 and 81-160 F_2 (W) individual plants of Population 1 F_2; (NT58WS$_6$#4 x ChallengerS$_6$-1) with NT58WS$_7$#4 susceptible as parent "P1" and ChallengerS$_6$-1 resistant as parent "P2"

Table 3. Genotypic data of 157 individual DNAs of F_2 Population 1 (P1 x P2); NT58WS$_6$#4 x Challenger S$_6$-1

Sweet corn lines	umc1042			bnlg1721			umc1149		
	Genotypic score			Genotypic score			Genotypic score		
	0	1	2	0	1	2	0	1	2
P1			1			1			1
P2	1			1			1		
F_1		1			1			1	
F_2; Homozygote (P1 type)			42			43			38
F_2; Homozygote (P2 type)	39			35			42		
F_2; Heterozygote		76			79			77	
Chi-square		0.27^{ns}			0.82^{ns}			0.26^{ns}	
χ^2 ($P<0.05$, df = 2)		5.99			5.99			5.99	

Association analysis of the three SSR markers to the *Ht* genes; *Ht1* and *HtN1* were performed among the 157 F_2 plants derived from Population 1 (P1 x P2). Results show that both umc1042 and bnlg1721 markers have highly significant F value of 27.27 and 32.18, respectively. Moreover, the coefficients of determination (R^2) were also reliable of 0.2616 and 0.2948, respectively (Table 4).

Based on these results, umc1042 and bnlg1721 were for marker assisted selection of sweet corn lines (Figure 5). The F_2 individual plants no. 105, 110 from Population 1 (P1 x P2) that showed homozygous with one DNA band similar to the DNA of the resistant male parents; ChallengerS_6-1 (P2), were selected. The F_2 heterozygous plants no. 85, 88, 130 and 133 with two DNA bands similar to the DNA of the female parents; NT58WS_6#4 (P1) x male parent; ChallengerS_6-1 (P2) were also selected. These 6 selected plants (F_2) were NCLB resistant with disease score 1, 2 and 3 in the field. These plants were used as male donor parents to backcross pollinated to recipient female (P1) elite inbred line with high consuming quality. The results show backcross population; 11 ears of BC_1F_1 was generated by NT58WS_6#4 x F_2 (Table 5).

Table 4. Association analysis between SSR markers and northern corn leaf blight disease score of 157 F_2 plants from Population 1; NT58WS_7#4 x ChallengerS_6-1

Gene	Chromosome	Marker	R- square	F-value	P-value
Ht1	2	umc1042	0.2616	27.27	< 0.0001**
Ht1	2	bnlg1721	0.2948	32.18	< 0.0001**
HtN1	8	umc1149	0.0092	0.71	0.4921^{ns}

Table 5. Marker assisted selection of F_2 plants for generating BC_1F_1 in Population 1 (W)**;** NT58WS_7#4 x Challenger S_6-1

F2 Plant No.	Disease score	umc1042		Bnlg1721		BC1F1 (ears)
		Genotype	Genotypic score	Genotype	Genotypic score	
105	3	homozygote	0	homozygote	0	2
110	3	homozygote	0	homozygote	0	2
85	1	heterozygote	1	heterozygote	1	2
88	1	heterozygote	1	heterozygote	1	1
130	2	heterozygote	1	heterozygote	1	3
133	2	heterozygote	1	heterozygote	1	1
Total						11

DISCUSSION

The study was able to identify ChallengerS_6-1, Sugar73S_7-18 and hA4135 as highly resistant lines to NCLB during the 2011 late rainy season inoculated trials. One line, NT58WS_6#4 was identified as highly susceptible. Three F_1 populations (Population 1, Population 2 and Population 3) were developed and used for association of markers closely linked to known *Ht* genes and their reaction to inoculation with *E. turcicum*. In a similar study, Welz and Geiger (2000) found that qualitative resistant genes *Ht2* and *HtN1* resistance to NCLB on chromosome 8 by using the diverse inbred susceptible crossed with resistant lines. The population sources were US Corn Belt germplasm (B52 x Mo17) and US Corn Belt crossed with African germplasm (Lo951 x CML202).

The 32 primers were deliberately chosen from Maize Genetics and Genomics Database (2012) specific to *Ht1*, *Ht2* and *HtN1* genes based on mapping population in the US. Three markers should polymorphism between the R and S lines and are possible markers for *Ht1* and *HtN1* selection among Thai sweet corn

germplasm. These markers could be used as markers assisted selection in sweet corn germplasm in Thailand. Especially, the resistant parent ChallengerS_6-1 which derived from an introduction of sub-tropical sweet corn variety from the US which is similar to the studied of Pataky *et al*. (2006). Therefore, some of sweet corn varieties in public and private sectors in Thailand might have the US germplasm introgression. It is a further implementation to validate the 2 markers; umc1042 and bnlg1721 on sweet corn germplasm and apply the marker assisted selection for NCLB disease resistance.

The association analysis of the three relevant SSR markers to the *Ht* genes; *Ht1* and *HtN1* performed among the 157 F_2 plants from population 1. Only two SSR markers: umc1042 and bnlg1721 closely linked to the same *Ht1* gene on chromosome 2 with genetic distance of 6.7 cM by MAPMAKER version 3.0 software. Yang (2010) reported the flanking markers umc1042 and bnlg198 linked to *Ht1* gene in cultivar Ent17 similar to the umc1042 that closely linked to the *Ht1* gene in ChallengerS_6-1. Moreover, Hooker (1963); Walz and Geiger (2000) reported that the *Ht1* gene was qualitative resistance and gene action was partial dominance. The Chi-square test of the genotypic data of 157 F_2 amplified by the three markers in Population 1 was non-significantly or qualitatively fit in 1:2:1 ratio (Table 2). For *Ht2* gene, Yin *et al*. (2003) reported that the SSR molecular marker; umc1149 was closely linked to the resistant gene *Ht2*. The marker umc1149 was also polymorphic for the plant material used in this study. However, association analysis indicate that association of this marker to resistance to NCLB was non-significant, R^2 = 0.0092 (Table 3). In conclusion, only SSR molecular markers; umc1042 and bnlg1721 were identified as specific markers for *Ht1* gene on chromosome 2 in the resistant sweet corn line**.**

To validate the use of umc1042 and bnlg1721 for marker assisted selection, F_2 lines from Population 1 were screened for their resistance or susceptibility to *E. turcicum*. The lines were selected based on their similar marker profile. In application of the relevant molecular markers; umc1042 and bnlg1721 to assist selection of sweet corn line, the combined results from plant molecular laboratory and field experiments were scrutinized simultaneously.

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