Genome-Wide Association Study (GWAS) Reveals an SNP Associated with Downy Mildew Resistance in Maize

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Abstract: Downy Mildew, caused by the fungus *Peronosclerospora* spp. is one of the most destructive diseases of maize and can cause severe damage in crops around the world, especially in tropical Asia. Host resistance is an effective mean of control DM and is reported to be controlled by many genes. Therefore, the identification of markers linked to the target quantitative trait loci (QTL) was required in a marker-assisted breeding program. In this study, Genome-Wide association study (GWAS) analysis using 262 maize inbred lines and 434,871 single nucleotide polymorphism (SNP) markers was performed to identify genomic regions and candidate genes associated with resistance to the DM. The result showed that one significant QTL was identified on chromosome 1 associated with the trait DM resistance, containing one candidate gene, Zm00001d029516, according to its function in plant protection mechanism. This QTL/SNP locus should be validated and will be useful for marker-assisted selection and for a better understanding of DM disease resistance in maize.

Keywords: *Preonosclerospora* spp, maize, quantitative trait loci (QTL), single nucleotide polymorphism (SNP)

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Introduction

Downy Mildew (DM), caused by the fungus *Peronosclerospora* spp. is one of the most important maize diseases and can cause severe damage in crops around the world, especially in tropical Asia (Kim *et al*., 2020; Rashid *et al*., 2018). In Asia, DM disease is caused by six pathogens, including sorghum DM, Philippine DM, Java DM, sugarcane DM, brown stripe DM, crazy top DM and Rajasthan DM (George *et al*., 2003; Yen *et al*., 2004). In Thailand, downy mildew has been found in several locations in maize growing areas and is caused by *P. maydis* (Java DM) (Janruang and Unartngam, 2018).

Downy mildew is very dangerous in tropical and subtropical lowland, midland, transitional and highland regions. The disease is mainly transmitted by oospores that survive in the soil, but it can also be transmitted from plant to plant by airborne conidia or by infected seed. It causes severe yield losses (up to 30% or more) in maize growing areas (Jeffers *et al*., 2000). Severe economic yield losses due to the various DM pathogens have been reported from the Philippines, Taiwan, Indonesia, Thailand, India, Japan, Australia, Venezuela, North America, Europe, West Africa, and other parts of the world (George *et al*., 2003; Jeffers *et al*., 2000).

Among the available options to control DM diseases, use of resistant cultivars is the most practical and cost-effective approach to disease controls (Ward *et al*., 1997). Therefore, the development and use of DM-resistant maize varieties is the most important way. However, breeding DM resistant lines using conventional method is challenging because accurate and efficient DM disease screening is limited to favorable weather condition with high disease pressure (Jadhav *et al*., 2019). This limits year-round selection of resistant genotypes through phenotypic screening. The use of markers linked to the target Quantitative Trait Loci (QTL) in a breeding program is a highly efficient and cost-effective tool to improve downy mildew disease resistance in maize. Therefore, the genomic regions or QTLs that contribute to resistance (DM) should be identified for direct use in the marker-assisted resistant breeding program. Many researchers investigated the inheritance of resistance to DM using QTL mapping and association mapping (Ashan *et al*., 2020; Jadhav *et al*., 2019; Jampatong *et al*., 2013; Lohithaswa *et al*., 2015; Rashid *et al*., 2018) and suggested that resistance to disease DM is a complex trait and is controlled by polygenes in nature (Nair *et al*., 2005; Nallathambi *et al*., 2010). George *et al*. (2003) studied on QTL mapping of resistance to diverse DMs including *P. maydis* in Asia regions using recombinant inbred lines (RILs) derived from Ki3 (resistant) and CML139 (susceptible) and RFLP (restriction fragment length polymorphism) markers. They identified six genomic regions or QTLs on chromosomes 1, 2, 6, 7 and 10 and found the most significant QTL on chromosome 6 (6.05) conferring resistance to DM pathogens. Phumichai *et al.* (2012) also reported that three SSR markers (bnlg1057, bnlg1138 and umc1033) on chromosome 1, 3, and 9 were observed in association with 48 SSR markers and downy mildew resistance (DM) using a set of 60 public and private maize inbred lines in Thailand. They identified significant SSR marker umc1033 linked with DM resistance on chromosome 9 (bin 9.02).

GWAS is a powerful tool for identifying genomic loci associated with phenotypic traits, and the main advantages of association mapping are higher mapping resolution, shorter research time, and the ability to identify a larger number of favorable alleles responsible for the trait of interest (Elshire *et al*., 2011; Ma *et al*., 2019). However, there has very limited information for GWAS analysis to identify the QTLs for DM disease including *P. maydis*. Therefore, the objective of this study was to identify the genomic regions and candidate genes associated with DM disease resistance in maize using SNPs markers in tropically adapted maize germplasm in Thailand.

Materials and Methods

1. Germplasm and Downy Mildew (DM) screening

In this study, a panel of 262 recombinant inbred lines was screened in the greenhouse with pots for the resistance against Suwan Farm isolate of DM disease by artificial inoculation. Of the 262 varieties, 71 sweet corn, 168 waxy corn and 23 field corn were included. One pot containing 5 plants was used as one replication for each variety. At 14 days after planting of the tested varieties, the DM disease leaves were collected from the infected fields and DM spores were obtained by washing the leaves. Experiments were laid out in a Randomized Complete Block Design with three replicates. Evaluation of DM was done according to Unartngam (2019). Broad-sense heritability was calculated according to the formula by Knapp *et al*., (1985).

2. Maize array Genotyping and SNP calling

Genomic DNA was extracted using the magnetic bead method (Xin and Chen, 2012). Five plants per sample were pooled and used for DNA extraction. The pooled panel of 262 maize inbred lines was genotyped using the Axiom™ maize 600 K genotyping array with 616,201 variants (Thermo Fisher Scientific). To determine the quality of the data, quality control (Q.C.) was performed with a call rate of 90% (517,293 SNPs) (Axiom genomic suit). Subsequently, SNP markers with more than 10% missing data, 20% heterozygosity, less than 5% minor allele frequency (MAF) were excluded from the dataset to obtain only bi-allelic sites (434,871 SNPs). These SNPs were used for GWAS analysis.

3. PCA, LD decay and population structure analysis

Principal component analysis (PCA) was also performed to determine the relationship between samples using the software TASSEL (Bradbury *et al*., 2007), removing SNPs with minor allele frequencies (MAFs) of 5% and limiting the number of components to three. Linkage disequilibrium (LD) of all SNP pairs on each chromosome was determined using the PopLDdecay (https://doi.org/10.1093/bioinformatics/bty875), with the following parameters: MAF>5%; Hardy–Weinberg P-value cut-off, 0; and percentage of genotyped lines >0.75. In addition, SNPs with high linkage disequilibrium (LD) were pruned using the in-depth-pairwise function implemented in PLINK (SNP window size: 50, shifted SNPS per step: 10, r2 thresholds: 0.1) (Niu *et al*., 2019) resulting in 160,991 SNP markers. This set of SNPs was used for population structure analysis using STRUCTURE (Evanno *et al*., 2005).

4. Genome-wide association analysis and Candidate genes association analysis

A genome-wide association study (GWAS) was then performed using GAPIT (Genomic Association and Prediction Integrated Tool) (Lipka *et al*., 2012) in R program (R Core Team, 2018). The p values of testing markers

and the associated markers are unified at each interaction (Liu *et al*., 2016). The threshold for a significant association was set based on the Bonferroni correction level of P-value ($P < 1/n$; n = total markers used). To determine the amount of variance explained by each significant SNP, an analysis of variance was performed for each significant SNP and the ratio between mean square of each SNP by error mean square was used to represent the variance percentage explained (Rossi *et al*., 2020). It was performed by single regression analysis in R program (R Core Team, 2018).

The available reference genome sequence of maize (B73) was used to identify candidate genes. SNP probe sequences of ~150 bp on Axiom® Maize 600K Genotyping Array (Thermo Fisher Scientific) were used as queries in a BLAST algorithm-based search against the reference genome sequence in MaizeGDB (http://www.maizegdb.org/gbrowse). The 200 bp source sequences of each significant SNP were used for BLAST against the ZmB73_RefGen_v4 genome sequence in MaizeGDB (Portwood *et al*., 2019). Within the local LD block of significant SNPs, the annotated genes that are likely involved in disease resistance were identified as the putative candidate genes.

Results

1. Phenotypic evaluation of DM disease

Phenotypes of the tested lines were evaluated against Suwan Farm isolate of DM disease. Significant phenotypic differences ($P \leq 0.01$) between the tested lines were observed in this experiment (Table 1), indicating high genetic variability in the collection of these inbred lines. The broad-sense heritability (H^2) for this experiment was 42.28 %. The percent disease index of the lines tested against Suwan Farm isolate ranged from 0-100%. The frequency distributions of the lines tested at DM for this isolate was shown in (Figure 1).

Table 1 Phenotypic variation for percent disease index (PDI) against Suwan Farm isolate of downy mildew disease on 262 recombinant inbred lines of maize.

Trait	Isolate	Mean	Min	Max	SD	CV%	H^2
DM	SW	44.06**	0	100	17.65	49.57	42.28%

DM: downy mildew; SW: Suwan Farm; Mean: average; Min: minimum; Max: maximum; SD: standard deviation; CV: coefficient of variation; H^2: heritability; **significant difference at $p<0.01$.

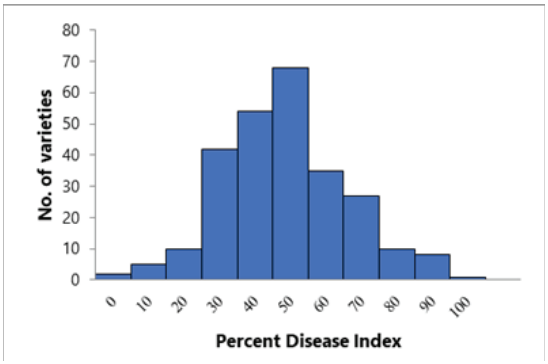

Figure 1 The frequency distribution of resistance to Suwan Farm isolate of downy mildew disease.

2. PCA and LD decay analysis

The genotype obtained from high-density maize SNP array was performed in a set of 262 maize lines to analyze a principle component analysis (PCA). Based on 434,871 SNP markersafter filtering with the parameters mentioned in materials and methods, PCA (total PCs=3) of all the genotype data was performed using the software PLINK. PC1 and PC2 explained most of the variation (16.6% and 12.1%, respectively) and were selected for visualization (Figure 2a). Significant clusters were observed for corn type as, sweet corn, waxy corn, and field corn (Figure 2b). The average heterozygosity of these varieties was 11.48 and ranged from 2.27 to 28.41. For LD decay analysis, all 434,871 filtered SNPs were used as input data for calculating the genome-wide LD in the associated panel. The total LD decay in the genome of the 262 maize associated panel was 206 Kb at a cut-off r^2 = 0.1, so a 200 kb region flanking the left and right sides of an SNP was defined as a QTL. At a cut-off of r^2 = 0.2, the mean length of LD decay rapidly decreased rapidly 48 kb (Figure 2c).

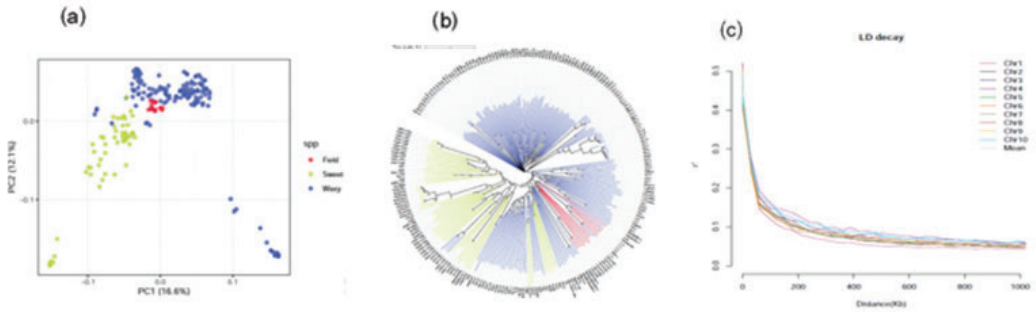

Figure 2 (a) Principle component analysis of 262 inbred lines of maize based on 434,871 SNP markers. (b) Clustering of 262 inbred lines based on 434,871 SNP markers. (c) LD, r^2 values against physical distance (Mb) between all SNP pairs on the same chromosome. The total LD decay across the genome of 262 maize associated panel using 434,871 SNP markers was 206 Kb at a cut-off r^2 = 0.1. At a cut-off of r^2 = 0.2, the mean length of the LD decay rapidly decreased to 48 kb.

3. GWAS Analysis for Marker Trait Associations

Association mapping was performed using fixed and random model Circulating Probability Unification (FarmCPU) method by integrating population structure and relatedness (kinship) within the tested panel, 434,871 SNPs with rare alleles (MAF < 5%) were excluded. Using the results presented in Manhattan plot and quantile-quantile (QQ) plot (Figure 3), the significant SNPs were observed at Bonferroni correction of $P \leq 2.29\times 10^{-6}$ ($P < 1/n$; n = total markers used). Using 434,871 SNPs markers, the most strongly associated SNP, AX-90669763 was identified on chromosome 1 with a P-value of $1.78\text{x}10^{-12}$, and a MAF, 0.12 for DM resistance (Table 2). The phenotypic variance explained for this SNP was 4.9%.

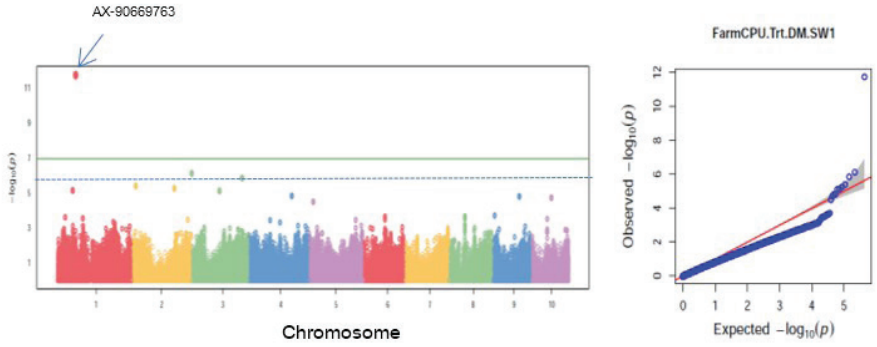

Figure 3 Manhattan plot and QQ plot resulting from the SNP-based GWAS for percent disease index (PDI) of downy mildew disease, Suwan Farm isolate. GWAS identified the significant highest SNPs markers that reached at the Bonferoni correction threshold of $P \leq 2.29\times 10^{-6}$ (5.60) using the FarmCPU model. X axis indicates physically mapped chromosomes. Y axis indicates significance as calculated by -log10 (P).

Table 2 SNP associated with DM resistance across Suwan Farm isolate.

Trait	SNP	Position (V4)	SNP Name	Chr:	P-value	Allele	MAF	R^2 (%)
SW	S1_74009842		AX-90669763	1	$1.78\text{x}10^{-12}$	A/G	0.123	4.90

DM: SW: Suwan Farm; SNP: Single nucleotide polymorphism; Chr: Chromosome; MAF: Minor allele frequency; R^2: Phenotypic variance explained

4. Candidate genes analysis

Based on the annotation information of maize B73 reference genome_V4, the candidate genes for resistance DM were identified by searching the MaizeGDB (B73 RefGen_v4) and found that the significant SNP on the chromosome 1 was located on the gene, Zm00001d029516, encoding the protein function of probable galacturonosyl transferase 11. Moreover, the allele effects of the significant SNP for the resistant trait DM in maize inbred lines were determined by boxplot analysis. For the QTL on chromosome 1, QTL1.1_AX-90669763_ position 74,009,842 (A/G, P-value=$1.78\text{x}10^{-12}$), the average disease index for allele with A was 41.69%, significantly lower than allele with G (68.85%) ($p \leq 0.05$, Figure 4).

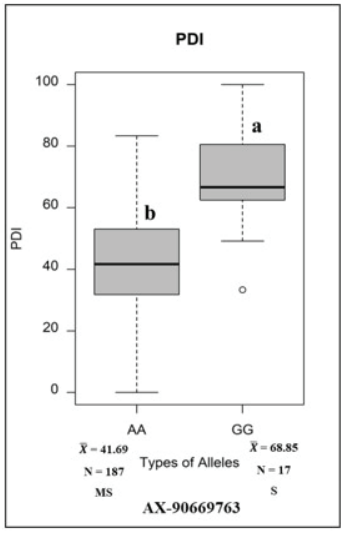

Figure 4 Allele effects of significant SNP for DM resistant trait in waxy maize, QTL1.1_AX-90669763_74009842 (A/G).

Discussion

Downy mildew of maize, caused by *Peronosclerospora* spp. is one of the most destructive diseases of this crop in tropical Asia (Yen *et al*., 2004). The use of resistant varieties is an effective way to control DM disease, and many researchers reported that resistance of DM is controlled by polygenes (Ward *et al*., 1997). In this study, the phenotypic responses of most inbred lines were susceptible to Suwan Farm isolate of DM disease. In additioin, the broad-sense heritability estimate of this evaluated experiment was intermidiate, at 42.28% (Table 1). Due to this level of heritability, a great effort must be made in a breeding program to accumulate a large number of resistance alleles in the germplasm (Brito *et al*., 2001; Rey and Lúquez, 2009). However, because the probability of selecting superior genotypes (i.e. inbred lines) is low, marker assisted selection (MAS) offers a strategy that could increase selection alleles.

There was very little previous research that had performed GWAS analyses for DM disease resistance including *P. maydis*. However, QTL mapping for DM resistance was performed by many groups on different DMs, including *P. maydis*, in different germplasms and environments. Janruang (2020) examined the GWAS analysis between 64 maize inbred lines and 35,404 SNP markers and identified three SNP markers on chromosome 2, 5, and 9 at Bonferroni threshold level of 3.5 but did not identify the candidate genes. According to the previous results, QTLs related to resistance to multiple DM diseases have been identified on chromosome 1, 2, 3, 6, 7, 9, and 10 using different markers in QTL mapping (Agrama *et al*., 1999; Ashan *et al*., 2020; George *et al*., 2003; Kim *et al*., 2020; Phumichai *et al*., 2012). In this study, only one significant SNP has been identified on chromosome 1 (bin 1.1) (Table 2, Figure 3) that is associated with DM disease resistance. Kim *et al*. (2020) investigated QTLs involved in resistance to *P. sorghi* (sorghum DM),

P. maydis (Java DM), and *Sclerophthora macrospora* (crazy top DM) using a recombinant inbred line (RIL) from a cross between B73 (susceptible) and Ki11 (resistant) and 234 sequence repeat (SSR) and restriction fragment length polymorphism (RFLP) markers. They identified seven QTLs with the phenotypic variation explained (PVE) of 0.47-12.47% on chromosome 2, 3, 6 and 9 and sixty-two candidate genes were detected. Ashan *et al.* (2020) observed seven QTLs controlling DM resistance genes on chromosome 1, 5, 6, and 10 in the whole genome map of F3 population by using 28 SSR markers with the PVE values ranged from 43.35 to 53.71%. Phumichai *et al.* (2012) also reported that three SSR markers (bnlg1057, bnlg1138 and umc1033) on chromosome 1, 3, and 9 were observed in association with 48 SSR markers and downy mildew resistance (DM) using a set of 60 public and private maize inbred lines in Thailand. Other studies have reported QTLs for resistance to *P. sorghi* (SDM) on chromosomes 2, 3, 4, 5, 6, 8 and 9 (Jadhav *et al*., 2019; Jampatong *et al*., 2013; Lohithaswa *et al*., 2015; Nair *et al*., 2005; Rashid *et al*., 2018; Sabry *et al*., 2006). In this study, the PVE of the significant SNP was very low, 4.9% (Table 2). Some researchers reported that the low PVE% indicated that MAS alone is not recommended for a breeding program (Knapp, 1998; Mohan *et al*., 1997). The best scheme should be used the combination of MAS with conventional selection methods (i.e: repeated selection) (Brito *et al*., 2001). Moreover, the most important genomic region for DM resistance including *P. maydis* was found on chromosome 2 (bin 2.01) (Kim *et al*., 2020), 6 (bin 6.05) (George *et al*., 2003) and 9 (bin 9.02) (Phumichai *et al*., 2012). Therefore, the significant association identified in this study seem to be novel and was not found in the previous QTL mapping studies on DM resistance including *P. maydis* (Table 2).

In addition, one candidate gene, Zm00001d029516, associated with disease resistance was identified at the associated locus on chromosome 1 (Table 3). This functional gene was associated with protein function of probable galacturonosyltransferase 11 (GAUT 11). GAUT11 play important roles in plant growth and development (Guo *et al*., 2021).

Conclusion

Based on this GWAS study, one significant QTL on chromosome 1 was identified to be associated with resistance of DM. Following the QTL region, only one candidate gene, Zm00001d029516, was identified based on its protein function that play important role in plant growth and development. In addition, the allelic composition of the QTL/SNP loci, AX-90669763 on chromosome 1, differed when comparing the allelic composition of the first five resistant lines with that of susceptible waxy maize, indicating its usefulness in pyramiding quantitative resistance to DM disease.

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