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Protocol Note

Identification of downy mildew resistance gene candidates by positional cloning in maize (*Zea mays* subsp. *mays*; Poaceae)[1]

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- *Premise of the study:* Positional cloning in combination with phenotyping is a general approach to identify disease-resistance gene candidates in plants; however, it requires several time-consuming steps including population or fine mapping. Therefore, in the present study, we suggest a new combined strategy to improve the identification of disease-resistance gene candidates.
- *Methods and Results:* Downy mildew (DM)–resistant maize was selected from five cultivars using a spreader row technique. Positional cloning and bioinformatics tools were used to identify the DM-resistance quantitative trait locus marker (bnlg1702) and 47 protein-coding gene annotations. Eventually, five DM-resistance gene candidates, including *bZIP34*, *Bak1*, and *Ppr*, were identified by quantitative reverse-transcription PCR (RT-PCR) without fine mapping of the bnlg1702 locus.
- *Conclusions:* The combined protocol with the spreader row technique, quantitative trait locus positional cloning, and quantitative RT-PCR was effective for identifying DM-resistance candidate genes. This cloning approach may be applied to other whole-genome-sequenced crops or resistance to other diseases.

Key words: downy mildew; maize; positional cloning; quantitative reverse-transcription PCR (RT-PCR); spreader row technique.

Downy mildew (DM), caused by several species of *Peronosclerospora* and *Sclerophthora*, is a major maize (*Zea mays* L.) disease in tropical and subtropical regions. The causal pathogens, also called DMs, are oomycetes (not true fungi) and are obligate parasites of higher plants. They can overwinter in soil or on plant debris and can eventually produce visible, characteristic "downy" hyphae on the leaves (Agrios, 1988). Owing to its widespread distribution and severe yield reductions, DM is one of the most destructive of the maize pathogens (Nair et al., 2005), and in Asia, it is the most important biotic stress affecting maize (Nagabhushan et al., 2014). Serious downy mildews of maize in Asia include *Peronosclerospora sorghi* (sorghum downy mildew; SDM) and *P. heteropogoni* (Rajasthan downy mildew; RDM) in India, *P. maydis* (Java downy mildew) in Indonesia, *P. zeae* (brown stripe downy mildew) in Thailand, *P. philippinensis* (Philippine downy mildew) in the Philippines, and *P. sacchari* (sugarcane downy mildew) in several Asian countries (George et al., 2003; Prasanna et al., 2010). Despite efforts toward the development of DM-resistant cultivars or seed treatment with metalaxyl fungicide, DM still emerges in localized areas as a severe pathogen (George et al., 2003). Inoculation methods for DM in maize that have been widely used are spraying inoculum and the spreader row technique (Shekhar and Kumar, 2012). Considering that DM is an obligate parasite, the spray inoculation requires abundant, synchronous sporulation within a short time, while the spreader row technique needs consecutive inoculations of the susceptible variety and the test variety (Craig, 1980; Shekhar and Kumar, 2012).

Forward genetics is an attractive tool to improve DM resistance in maize breeding. Several quantitative trait loci (QTL) studies have identified quantitative DM-resistance genes by linkage mapping in maize (Agrama et al., 1999; George et al., 2003; Nair et al., 2005; Sabry et al., 2006; Jampatong et al., 2012). Although the QTLs and population sample sizes were diverse, the QTL analysis located DM resistance–related genomic regions on chromosomes 1, 2, 3, 6, 7, and 10 (George et al., 2003; Jampatong et al., 2012). Because the identification of QTLs so far has been limited by high cost and poor resolution, a combined protocol with positional or map-based cloning is a powerful approach to identify resistance genes against DM (Anderson et al., 2011; Phumichai et al., 2012; Franchel et al., 2013). Positional cloning is a useful tool to identify

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a gene for a specific phenotype in the absence of sequence information about target products. In view of this advantage, positional cloning can be applied to cross-species hybridization, mutation screening, and CpG island identification (Gallavotti and Whipple, 2015). However, positional cloning is a tedious process that requires several steps. Above all, population mapping or fine mapping of target loci is an integral part of positional cloning (Anderson et al., 2011). Additionally, positional cloning depends on the presence of appropriate genetic markers that have a traceable heredity in company with the target traits (Martin and Hine, 2015). Due to the large genome size of maize, positional cloning in maize is an inefficient approach (Gallavotti and Whipple, 2015). Simko et al. (2013) developed recombinant inbred lines (RIL) and identified DM-resistant QTLs (*qDM2.1* and *qDM5.1*) in lettuce cultivars. Recently, Gallavotti and Whipple (2015) reported successful positional cloning by *sh1* mutant screening and developed molecular markers for fine mapping. However, their methods included creating a backcross population and an F_2 population.

In the present study, we developed a DM inoculation technique for use in the field. For easy inoculation, spreader rows were replaced with pots of susceptible maize. After QTL marker screening and positional cloning, without creating both a backcross and F_2 population, DM-resistant gene candidates were identified by sequence information and quantitative reverse-transcription PCR (RT-PCR).

METHODS AND RESULTS

***Plant materials*—**Five maize nested association mapping (NAM) parent lines—B73, CML228, CML270, Ki3, and Ki11—were used to identify DM resistance–related genes. The NAM parent lines, from the U.S. Department of Agriculture (USDA), were grown at a research field near Phnom Penh, Cambodia. For positional cloning, 3-wk-old leaves were harvested and stored at −80°C until DNA extraction for the QTL analysis. For analysis of transcript accumulation in healthy or DM-infected maize, 6-wk-old leaves were harvested and

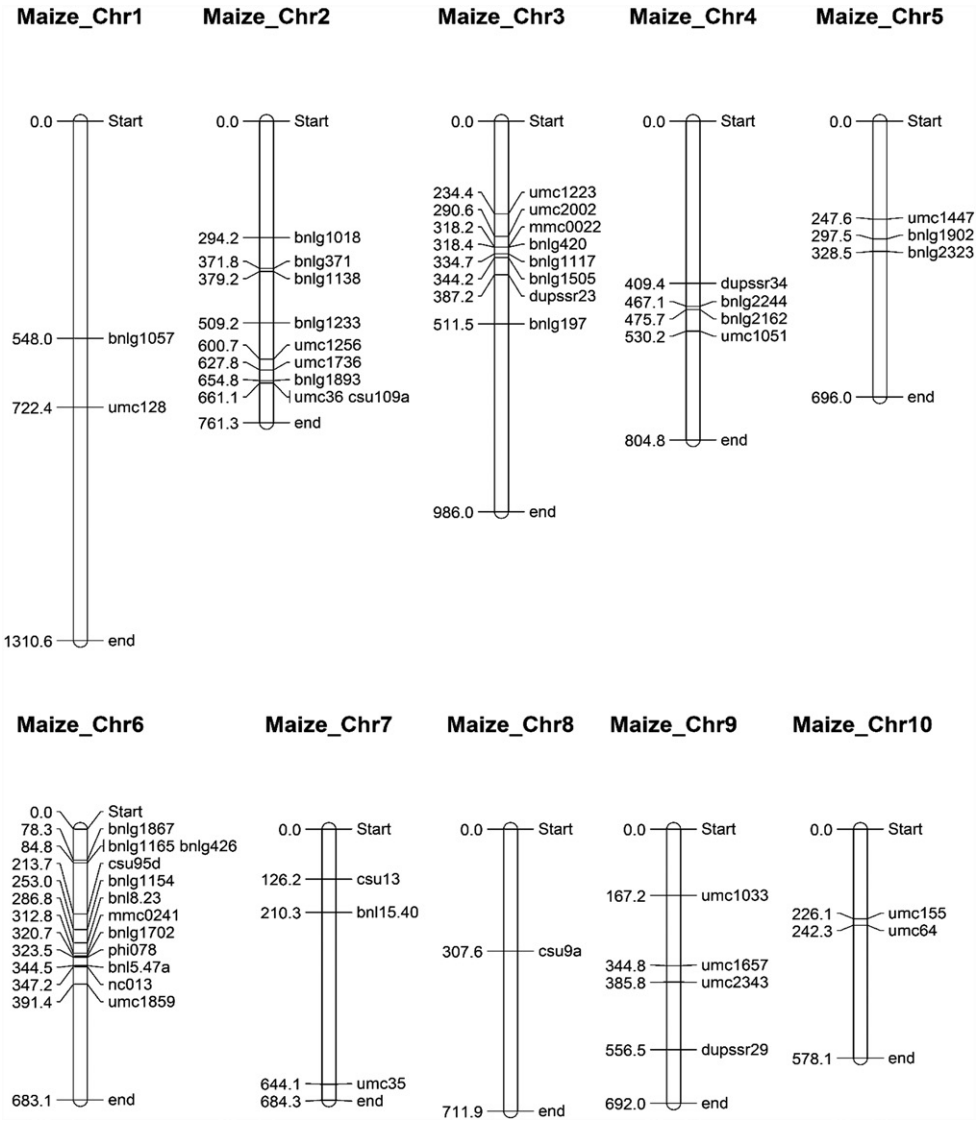


Fig. 1. Molecular linkage map of 59 SSR RFLP loci and location of QTLs. Numbers to the left of the chromosomes indicate the cumulative distance of the consensus map in cM. The consensus map of the IBM2 2008 neighbors was used with MapChart. The DM-resistance QTLs were obtained from the literature (Agrama et al., 1999; George et al., 2003; Nair et al., 2005; Sabry et al., 2006; Jampatong et al., 2012; Phumichai et al., 2012; Nagabhushan et al., 2014).

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immersed in RNA*later* solution (ThermoFisher Scientific, Waltham, Massachusetts, USA) to stabilize the mRNA until extraction.

***Downy mildew inoculation and evaluation*—**Maize leaves were inoculated with downy mildew from infected plants using the modified spreader row technique described by George et al. (2003). The inoculation was performed twice: in April and September. Susceptible genotypes B73 and CML270 were used in the spreader rows. The spreader rows consisted of two rows with 50 × 25 cm spacing, with 20 seeds planted after every 10th row of the test entries (Appendix S1). Seeds of the spreader plants germinated after 7 d, then DW-infected maize was placed in plastic pots in the middle of each experimental block for 2 wk to infect the spreader plants. After removing the DM-infected maize pot, test genotypes were planted with 50 × 25 cm spacing between the spreader rows. After the emergence of the test genotype, the disease reaction was monitored and scored every 7 d for 6 wk. DM resistance was evaluated at weeks 4 and 6 during the scoring period according to the scale used by Craig (1980) and Nagabhushan et al. (2014): 0% infection (no symptom) = highly resistant (HR), 1–10% infection = resistant (R), 11–25% infection = moderately resistant (MR), 26–50% infection = moderately susceptible (MS), 51–75% infection = susceptible (S), and 76–100% infection = highly susceptible (HS).

The DM inoculations were confirmed to result in 100% infection of the susceptible cultivars, B73 and CML270. DM infection began 3–4 wk post-inoculation, then spread rapidly throughout the field. The infected maize stopped growing 6 wk after inoculation, then withered (Appendix S2). Although a few infected maize plants grew after the DM inoculation, we observed severely suppressed stamen and pistil development. In the present study, lines CML228, Ki3, and Ki11 had low to very low DM incidence, ranging from 0% to 5% in the April test and from 22.5% to 25% in the September test (Table 1).

According to the weather records in Phnom Penh, Cambodia, in 2015, temperatures were high and humidity low in April to June, while temperatures were low and humidity high in September to October (Appendix S3). Because slightly low temperatures and high humidity are favorable for the development of DM (Shekhar and Kumar, 2012), we inferred that the lower DM incidence in the April test was related to the high temperatures and relatively low humidity compared with the more favorable conditions later. Eventually, CML228, Ki3, and Ki11 were determined to have a high DM resistance in the two independent tests. By contrast, we identified B73 and CML270 as susceptible cultivars; B73 and CML270 were 100% infected by DM at 6 wk after inoculation in both the April and September tests.

***DNA isolation and SSR screening for positional cloning*—**Genomic DNA was extracted from 0.5 g of fresh leaves using the cetyltrimethylammonium bromide (CTAB) method described by Kim (2013), including washing steps with sodium acetate (Appendix 1). Because QTL marker screening with genomic DNA is not influenced by plant maturity during DM infection, we extracted genomic DNA from 3-wk-old leaves to shorten the experimental period. Simple sequence repeat (SSR) markers reported to be linked to DM-resistant QTLs were selected from the MaizeGDB (http://www.maizegdb.org) based on preliminary publications (Fig. 1). A total of 48 polymorphic SSR markers or restriction fragment length polymorphism (RFLP) probes from all 10 chromosomes were used for screening. The amplification was performed using a GeneTouch thermal cycler (Bioer Technology, Hangzhou, Zhejiang Province, China) with abm *Taq* DNA polymerase (Applied Biological Materials, Richmond, British Columbia, Canada). The PCR program consisted of one cycle of 10 min at 94°C for the initial denaturation; 33 cycles of 1 min at 94°C, 30 s at the proper annealing temperature, and 1 min at 72°C; and final elongation for 4 min at 72°C. The PCR products were separated by electrophoresis in a 1.5% agarose gel with 1× Tris-acetate-EDTA (TAE) buffer and visualized using ethidium bromide staining. The DM resistance–related region was confirmed by the presence of the PCR bands for the resistant (HR) cultivars and their absence for the susceptible (HS) cultivars. The 59 QTL-linked markers (48 loci) used to identify the DM resistance–related regions and the primer information are shown in Appendix S4. The QTL-linked markers were distributed on the entire chromosome; however, 30 of 48 loci were located on chromosomes 2, 3, and 6 (Fig. 1). PCR amplification revealed 22 polymorphic QTL-linked markers in five maize cultivars (Appendix S5). After the PCR screening with the QTL-linked markers, the polymorphism associated with bnlg1702 was found to be identical to the results in Table 1. The bnlg1702 marker amplified a PCR product of approximately 370 bp in CML228, Ki3, and Ki11, but not in B73 and CML270. MaizeGDB was used to find genetic information for SSR marker bnlg1702 on chromosome 6. Sequence information and predicted transcripts were obtained using EnsemblPlants (http://plants.ensembl.org/index.html). According to MaizeGDB information, bnlg1702 is flanked by umc2321 and AY110873, and the genetic location is estimated to be between positions 146,503,155 and 147,912,501 on chromosome 6 based on the IBM2 2008 neighbors. QTL-linked markers umc2321 and AY110873 were also used in the PCR screening of the five NAM parent lines (Appendix S5). Although polymorphisms of umc2321 and AY110873 did not exactly match bnlg1702, umc2321 and AY110873 yielded different PCR products between DM-susceptible and DM-resistant cultivars. An approximately 1.4-Mb fragment was identified for the DM resistance–related region. In all, 47 protein-coding gene annotations were obtained using the MAKER gene annotation pipeline (Cantarel et al., 2008) and contained 63 predicted transcripts for this fragment. Gene-specific primer sets were designed based on the 63 predicted transcripts using Primer3 (Rozen and Skaletsky, 1999) to validate the actual expression of each transcript in healthy or DM-infected maize plants (Appendix S6).

TABLE 1. Disease reactions at four and six weeks after inoculation of five maize cultivars against downy mildew in Phnom Penh, Cambodia.[a,b]

	April–June		September–October	
Cultivar	Week 4	Week 6	Week 4	Week 6
Susceptible genotype				
B73	MS (50%)	HS (100%)	HS (100%)	HS (100%)
CML270	HS (100%)	HS (100%)	S (75%)	HS (100%)
Test genotype				
CML228	HR (0)	HR (0)	MR (20%)	MR (25%)
Ki3	HR (0)	HR (0)	MR (16.6%)	MR (22.2%)
Ki11	HR (0)	R (5%)	R (10%)	MR (25%)

[a]Scale for host reaction: 0%, highly resistant (HR); 1–10%, resistant (R); 11–25%, moderately resistant (MR); 26–50%, moderately susceptible (MS); 51–75%, susceptible (S); 76–100%, highly susceptible (HS).

[b]Downy mildew incidence (%) = (number of downy mildew–infected plants/total number of plants) × 100.

***Quantitative RT-PCR*—**Total RNA was extracted from 6-wk-old leaves from healthy or infected NAM parent lines by using TRIzol (Invitrogen, Carlsbad, California, USA). Because the highest DM incidences were observed in 6-wk-old plants, transcripts of the DM resistance–related genes may be abundant in leaf samples from infected 6-wk-old plants. For RT-PCR, cDNA was synthesized from 1 μg of total RNA using a Power cDNA synthesis kit (iNtRON Biotechnology, Seongnam, Gyeonggi, Korea) according to the manufacturer's instructions. Diluted cDNAs were used as a template for quantitative RT-PCR using a gene-specific primer set (Appendix S6) and the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, California, USA) using the EverGreen 2× qPCR MasterMix (Applied Biological Materials). The amplification was performed as follows: initial denaturation at 95°C for 10 min, followed by 44 cycles of 15 s at 95°C and 1 min at 56°C. Amplicon specificity was examined using a melt curve analysis from 65°C to 95°C. Three replicates of real-time PCR experiments were performed for each predicted transcript. The relative gene expression compared with the control plant (healthy B73) was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The transcripts significantly upregulated during DM infection in resistant maize cultivars—CML228, Ki3, and Ki11—were selected as putative DM resistance–related transcripts by RT-PCR. Expression of the 63 predicted transcripts was profiled by RT-PCR; however, four predicted transcripts did not amplify in healthy or DM-infected maize plants (Appendix S7). Nine putative DM-resistance transcripts were primarily selected, and eventually five transcripts were identified as DM-resistance candidate genes after normalization with the expression level of healthy B73 cultivars (Fig. 2). As compared with transcripts in infected B73, the average transcript accumulation ratios in the five selected candidates were calculated, and then the relative ratio was expressed as a percentage of 100% for B73, 90.5% for CML270, 388.7% for CML228, 781.9% for Ki3, and 329.6% for Ki11 (Fig. 2). According to the results in Table 1, Ki3 was determined to be the most DM-resistant maize cultivar by phenotyping with the spreader row technique. Transcript abundance in infected maize plants was much greater than the average transcript accumulation in healthy B73. The expression ratio for each infected maize cultivar as compared with healthy samples was determined to be 132.5% for B73, 92.7% for CML270, 1049.6% for CML228, 1004.6% for Ki3, and 2348.9% for Ki11.

***Identification of DM-resistant gene candidates*—**Five putative DM-resistant candidate genes were identified (Table 2). GRMZM2G042154 and GRMZM2G098209 encoded uncharacterized proteins, but GRMZM2G042154

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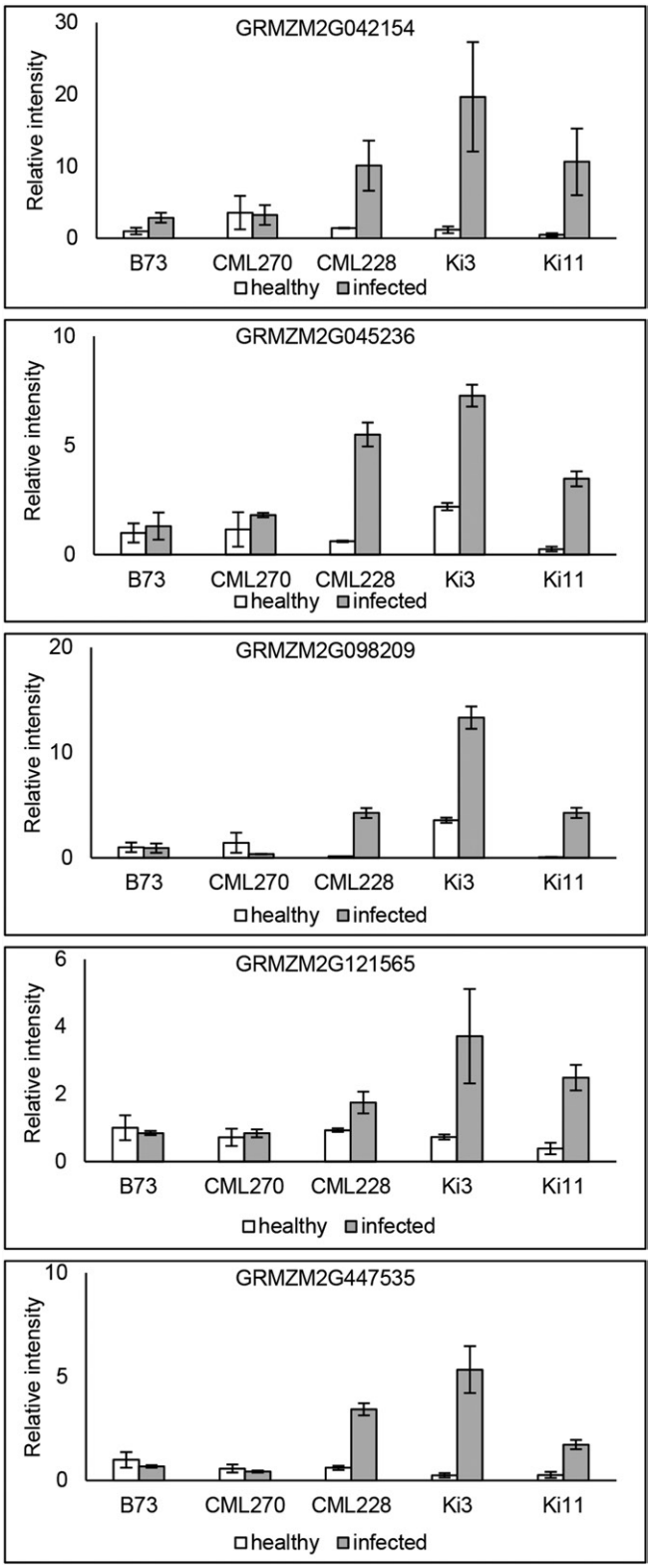


Fig. 2. Expression profiles of candidate genes for downy mildew resistance determined using quantitative RT-PCR. Total RNA from leaves from heathy or downy mildew–infected 6-wk-old maize plants was used to synthesize cDNA. The expression intensity was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), with B73 healthy as the control. The GRMZM2G042154, GRMZM2G045236, GRMZM2G098209, GRMZM2G121565, and GRMZM2G447535 transcripts were annotated as coding for uncharacterized protein (transporter), the bZIP transcription factor (*bZIP34*), an uncharacterized protein, brassinosteroid insensitive 1-associated receptor kinase 1 (*Bak1*), and pentatricopeptide repeat-containing protein (*Ppr*), respectively.

was classified as a transporter because it contained a transmembrane domain (NTR1/PTR family). The putative transcript GRMZM2G045236 encoded the bZIP transcription factor (bZIP34). GRMZM2G121565 was identified as a putative Brassinosteroid Insensitive 1-Associated receptor Kinase 1 (BAK1), and GRMZM2G447535 corresponded to pentatricopeptide repeat-containing protein (Ppr). In the microarray by Wei et al. (2012) to profile the expression of 125 maize bZIP genes after pathogen infection, the expression of *bZIP34* in the microarray did not change after infection by various fungi. On the other hand, in the present study, GRMZM2G045236 in resistant cultivars was expressed in response to DM infection (Fig. 2). Because expression profiling by microarray is only estimated by prediction and comparison, we expected that *bZIP34* in the resistant maize might be expressed following pathogen attack, especially after DM infection. BAK1, a leucine-rich repeat receptor kinase that is known as a key component in brassinosteroid signaling (Nam and Li, 2002; Shi et al., 2013), forms a complex with flagellin sensing 2 (FLS2) and acts as a positive regulator in brassinosteroid signaling and as an initiation signal of inherent immunity (Chinchilla et al., 2007). Previously, Shi et al. (2013) demonstrated that BAK1 is related to powdery mildew resistance using a *bak1* mutant in *Arabidopsis*. Although maize *Bak1* is involved in powdery mildew resistance, transcript GRMZM2G121565 might play a role in resistance against DM infection. *Ppr* is a plant resistance gene analog and an R gene candidate (Sekhwal et al., 2015). Considering that the grape *Ppr* gene is upstream of the DM resistance gene, *Rpv12*, which was identified by QTL analysis (Venuti et al., 2013), the *Ppr* gene has a high possibility for linkage with *Rpv12*. Laluk et al. (2011) demonstrated the *Arabidopsis* mitochondrial-localized pentatricopeptide repeat protein (PGN) functions in the defense response against fungi and salt stress. It should be noted that the maize *Ppr* gene was identified as *Rf2* (accession no. AAC49371) on chromosome 9 (Cui et al., 1996; Sekhwal et al., 2015). Because, according to the QTL screening (Fig. 1, 2), *Ppr* gene GRMZM2G447535 is located on chromosome 6, another maize *Ppr* gene may be involved in DM resistance. Interestingly, indel polymorphisms were found in both susceptible B73 and resistant Ki3 and Ki11 with regard to sequence alignment (Appendix S8). The indel region requires further study to develop a molecular marker for DM resistance.

In the present study, we selected DM-resistant maize cultivars using the spreader row technique. As compared with artificial inoculation (inoculum spraying), our new protocol, which improves on the spreader technique, includes an easy inoculation method and is time- and labor-efficient. Rapid evaluation of DM resistance is an outstanding advantage of this protocol because infection was achieved within 3 or 4 wk after inoculation. In a previous study to evaluate 40 maize cultivars, including five NAM parent lines, for DM resistance and incidence, B73 and CML270 were determined as susceptible, while Ki3, Ki11, and CML228 were determined as resistant (Kim et al., 2016). Congruently with our DM phenotyping, B73 and CML270 were found to be susceptible. Additionally, we suggest that the choice of a susceptible cultivar as a spreader plant is a crucial step for the rapid evaluation of DM resistance. Positional cloning with the QTL screening provides a clue to the DM resistance region that is approximately 1.4 Mb long. The predicted transcripts obtained using bioinformatics tools enabled the design of gene-specific primers and led to the identification of DM-resistant gene candidates using quantitative RT-PCR without fine mapping of the bnlg1702 locus. The candidates, namely, *bZIP34*, *Bak1*, *Ppr*, and genes encoding uncharacterized proteins, appear to be involved in DM resistance. However, further detailed research, including an expression study or marker screening of a segregating population and an analysis of a Mu insertion mutant will be needed to obtain definite evidence and to completely verify the DM-resistance mechanism. Even so, our protocol provides useful genetic information to improve the molecular breeding of maize.

CONCLUSIONS

Identification of DM-resistant genes is one of the most important research topics for breeding DM-resistant maize. In this study, we used a combined protocol involving the spreader row technique, QTL screening with phenotyping, and quantitative RT-PCR to identify DM-resistance gene candidates. Our new strategy included field selection using the spreader row technique

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Table 2. List of DM-resistance candidate genes selected using positional cloning and quantitative RT-PCR.

No.	Transcript ID[a]	Type[b]	Length (bp)	Predicted protein size (aa)	Name
11	GRMZM2G042154	T01	1057	303	Uncharacterized protein (transporter)
18	GRMZM2G045236	T01	2668	654	Putative bZIP transcription factor (bZIP34)
22	GRMZM2G098209	T01	2247	649	Uncharacterized protein
30	GRMZM2G121565	T02	2212	630	BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1
44	GRMZM2G447535	T01	3343	608	At4g14050, mitochondrial (LOC103630373)

[a] Predicted transcript ID obtained from EnsemblPlants (http://plants.ensembl.org/index.html).
[b] Possible coding sequence variant of predicted transcript.

without mapping or segregation population preparation, identification of the DM-resistance region using positional cloning and bioinformatics tools, and expression-level profiling using quantitative RT-PCR without fine mapping. As whole-genome information becomes available for other crops, our novel protocol should be applicable to other crops and other diseases when suitably customized.

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Appendix 1. Steps in combined protocol to screen for downy mildew resistance candidate genes. All procedures are depicted in Appendix S9.

I. Screening of maize genotype against downy mildew (DM) using spreader row method

1. Spreader plants (S), consisting of two rows with 50 × 25-cm spacing and 20 seeds, were sown before planting seeds of the test entries. Twenty seeds of spreader plants (S), consisting of two rows with 50 × 25-cm spacing, were planted before planting of the test entries.
2. DM-infected maize plants were collected in the research field and transplanted into plastic pots for easy movement.
3. After the emergence of the spreader plants, DM-infected maize plants in plastic pots were placed in the middle of each experimental block.
4. DM symptoms normally appeared within 2 wk after infection.
5. Pots of DM-infected maize plants were removed from the experimental block and planted with 50 × 25-cm spacing between the spreader rows.
6. The disease reaction was monitored and scored every 7 d for 6 wk.
7. The DM resistance was recorded for each plant at weeks 4 and 6 during the scoring period.
8. The disease severity ratings were as follows:

   DM resistance: 0% infection (no symptom) = highly resistant (HR), 1–10% infection = resistant (R), 11–25% infection = moderately resistant (MR), 26–50% infection = moderately susceptible (MS), 51–75% infection = susceptible (S), 76–100% infection = highly susceptible (HS)

II. Quantitative trait loci (QTLs) screening for positional cloning

1. Genomic DNA extraction. Extract genomic DNA using the cetyltrimethylammonium bromide (CTAB) method with a minor modification as described by Kim (2013).

   1-1. Prepare sufficient quantity of CTAB buffer (100 mM Tris, 20 mM EDTA [pH 8.0], 1.4 M NaCl, 2% [w/v] CTAB).

   1-2. Add fresh β-mercaptoethanol (200 µL β-mercaptoethanol per 100 mL buffer) and 0.1% (w/v) polyvinylpyrrolidone (PVP) into the CTAB buffer. Incubate the extraction buffer in a water bath at 65°C before use.

   1-3. Cool mortar and pestle by adding liquid nitrogen. Chop 0.5 g of fresh maize leaves into the liquid nitrogen using scissors, or add leaves to mortar and cut them into small slices, add liquid nitrogen and grind to a fine powder.

   1-4. Add frozen leaf powder to 5 mL of pre-heated buffer in a 50-mL disposable polypropylene tube and mix well to remove lumps. Add 10 µL of RNase (30 mg/mL) into the tube if desired.

   1-5. Incubate at 65°C for 1 h with several inversions.

   1-6. Cool the samples at room temperature for 2–3 min, then centrifuge at 10,000 × *g* for 2 min and transfer supernatant into a new tube.

   1-7. Transfer the supernatant, add an equal volume of chloroform–isoamylalcohol (24 : 1) and mix gently for 10 min.

   1-8. Centrifuge at 10,000 × *g* for 10 min. Carefully transfer the aliquot into a new tube.

   1-9. Add an equal volume of cold isopropyl alcohol and mix gently by inverting. Incubate at −20°C for 30 min or overnight.

   1-10. Use a blue pipette tip or glass bar to hook up the DNA and transfer to a new tube.

   1-11. Wash the DNA with washing buffer 1 (76% ethanol, 0.2 M sodium acetate) while shaking gently at room temperature.

   1-12. Allow the tube contents to settle for 3 min and discard washing buffer 1.

   1-13. Add washing buffer 2 (76% ethanol, 10 mM ammonium acetate) and shake the DNA gently at room temperature for 10 min.

   1-14. After centrifugation at 10,000 × *g* for 10 min, discard supernatant.

   1-15. Add 70% of ethanol to rinse the pellet and centrifuge again for 10 min.

   1-16. Air dry briefly (10 min) and remove the remaining washing buffer.

   1-17. Resuspend the pellet in 100 µL TE or H_2O.

2. Dilute DNA to final concentration of 50 ng/µL.
3. Choose SSR markers reported to be DM resistance–related QTLs from MaizeGDB (www.maizwgdb.org) based on preliminary publications.
4. Set PCR program as follows: one cycle for 10 min at 94°C for the initial denaturation; 33 cycles for 1 min at 94°C, 30 s at the proper annealing temperature, 1 min at 72°C; and final elongation for 4 min at 72°C. Separate PCR products by electrophoresis in a 1.5% agarose gel with 1× Tris-acetate-EDTA (TAE) buffer and stain using ethidium bromide.
5. After the QTL screening, obtain information about the locus position using MaizeGDB. Usually, MaizeGDB provides genetically mapped position information based on AGI's B73 RefGen version2 sequence.
6. Obtain sequence information and predicted transcripts using EnsemblPlants (http://plants.ensembl.org/index.html).
7. Use estimated loci to annotate protein-coding genes with the MAKER gene annotation pipeline (Holt and Yandell, 2011). However, each protein-annotated gene may possess one or more predicted transcripts.

III. Quantitative screening of maize genotypes against DM resistance

1. Gene-specific primer sets were designed using Primer3 (Rozen and Skaletsky, 1999) based on predicted transcripts for quantitative real-time PCR. Gene-specific primers were designed to amplify a 75–150-bp PCR product for higher efficiency. However, PCR products at least 100 bp long were required to easily distinguish them from potential primer dimers.
2. Gene-specific primers preferably have a GC content of 50–60% without long (>4) repeats of single bases. For efficient real-time PCR reactions, primers were adapted to an annealing temperature with similar melting temperature between 50°C and 65°C.

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3. Total RNA was extracted from leaf samples from healthy or DM-infected maize plants.
   3-1. Sterilize mortar, pestle, and all plastic ware with 0.1% diethyl pyrocarbonate (DEPC) water.
   3-2. Cool sterilized mortar and pestle by adding liquid nitrogen, then add fresh maize leaf samples (1 g) and grind to a fine powder.
   3-3. Add Trizol (10 mL) and incubate the mixture at room temperature for 5 min.
   3-4. Centrifuge sample at 10,000 × *g* for 10 min.
   3-5. Transfer supernatant to a new tube and add 2 mL of chloroform with shaking for 15 s, then incubate at room temperature for 2–3 min.
   3-6. Centrifuge sample at 10,000 × *g* for 15 min, then carefully transfer the aqueous phase to a new tube.
   3-7. Add 2.5 mL of isopropyl alcohol and 2.5 mL of high salt precipitation solution (0.8 M sodium citrate, 1.2 M NaCl) and mix well.
   3-8. Incubate sample at room temperature for 10 min and then centrifuge for 10 min.
   3-9. Discard the supernatant and wash the pellet with 10 mL of 75% ethanol (diluted with 0.1% DEPC).
   3-10. After centrifugation for 5 min, discard the supernatant.
   3-11. Air dry the pellet for 10 min and then add RNase-free water for re-suspension.
4. Total RNA was used to synthesize cDNA.
   4-1. Bring 1 µg of total RNA (in an RNase-free tube) to a final volume of 9.5 µL with RNase-free water.
   4-2. Add 1 µL of 0.2 mM oligo$(dT)_{15}$ and incubate mixture at 70°C for 5 min.
   4-3. Prepare the mixture (0.5 µL of AMV reverse transcriptase [10 U/µL], 1 µL of RNase inhibitor [10 U/µL], 2 µL of dNTP [each 2.5 mM], 2 µL of DTT [0.1 M]) and mix with the RNA samples.
   4-4. Incubate the sample at 42°C for 1 h and then heat to 70°C for 5 min to terminate the reverse transcription.
5. After dilution of the cDNA, the real-time PCR was carried out as follows: one cycle of 10 min at 94°C for the initial denaturation; 33 cycles of 1 min at 94°C, 30 s at the proper annealing temperature, and 1 min at 72°C; and final elongation for 4 min at 72°C.
6. The expression level was normalized with untreated WT (healthy B73) using the $2^{-\Delta\Delta Ct}$ method.

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