Mapping of *HtNB*, a gene conferring non-lesion resistance before heading to *Exserohilum turcicum* (Pass.), in a maize inbred line derived from the Indonesian variety Bramadi

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**ABSTRACT.** The gene *HtNB* confers non-lesion resistance to the fungal pathogen *Exserohilum turcicum* in maize. To map this gene, we developed two F₂ populations, P111 (resistant line) x HuangZao 4 (susceptible line) and P111 x B73 (susceptible). *HtNB* was located on chromosome 8.07 bin, flanked by MAC216826-4 and umc2218 at distances of 3.3 and 3.4 cM, respectively. *HtNB* appears to be a new gene responsible for resistance to northern corn leaf blight. Functions of the genes in the region between umc1384 and umc2218 were predicted. In addition, several genes were found to be related to disease resistance, such as the genes encoding Ser/Thr protein kinase and protein-like leaf senescence.

**Key words:** Corn; *Exserohilum turcicum*; Northern corn leaf blight; Gene mapping; SSR
INTRODUCTION

Northern corn leaf blight (NCLB), caused by Setosphaeria turcica (Luttrell) Leonard & Suggs (anamorph Exserohilum turcicum (Pass.) Leonard & Suggs), is the most common disease of maize and can result in yield reduction of 15-30% (data from http://maizedoctor.cimmyt.org) or greater, depending on the stage of maize development when the initial infection occurs (Raymundo and Hooker, 1981). As a foliar disease, NCLB prevails in humid areas with moderate temperatures (Levy and Cohen, 1983).

Monogenic resistance, conferred by the so-called Ht genes, results in distinct reactions to avirulent races of E. turcicum. The Ht1, Ht2, and Ht3 genes confer a chlorotic lesion inhibiting sporulation (Hooker, 1963, 1977, 1981), while HtN extends the incubation period, resulting in delayed symptoms until anthesis (Gevers, 1975; Raymundo et al., 1981). The recessive gene ht4 was found during recurrent selection of maize synthetic BS19, which expressed circular chlorotic halos of about 1 cm in diameter (Carson, 1995). Recently, a novel dominant gene, HtP, and a recessive gene, rt, conferring resistance to E. turcicum were mapped to chromosome 2.08 and 3.06 bin, respectively (Ogliari et al., 2007).

Polygenic resistance to NCLB is expressed as a reduction in the development of disease severity, including a decrease in lesion number and size, and an increase in incubation period and latency period (Raymundo and Hooker, 1982; Li and Liu, 1984; Smith and Kinsey, 1993). QTLs conferring incubation period and area under the disease progress curve have always been detected on chromosome 8L (Dingerdissen et al., 1996; Freymark et al., 1994; Schechert et al., 1999). QTLs for disease severity are found in all chromosomes, except for chromosome 7 and 10 (Welz et al., 1999; Zheng et al., 2007). QTLs conferring lesion length, lesion width, and lesion area are distributed on chromosomes 1, 3, 5, and 8 (Huang et al., 2002). Additionally, a region in tasselseed2 (ts2) has been proposed to participate in jasmonic acid signaling and play an important role in resistance to NCLB (Kolkman et al., 2009).

Resistant cultivars have been wildly used to control NCLB. E28 contains the gene Ht1, which confers resistance to E. turcicum, and was derived from the cross of Lv-9kuan x A619Ht1, followed by three backcrosses with Lv-9kuan and by gradual selection by self-pollination (Wu et al., 1996). Great effort has been made to investigate the disease resistance of widely used maize lines in China, and 87.3-94.4% of the lines were found to be moderately sensitive or highly sensitive to this disease (Gao et al., 1997; Zhao, 2000). An Indonesian landrace called “Bramadi” contains a dominant gene named HtNB on chromosome 8, conferring a non-lesion resistance at the flowering stage. HtNB has an independent hereditary pattern to Ht1, Ht2, and Ht3, and has a dominant epistatic effect, which demonstrates that the non-lesion resistance could inhibit the expression of the chlorotic-lesion phenotype (Xu et al., 1987).

In this study, the resistant gene in maize inbred line P111, derived from “Bramadi”, was confirmed in the HtNB region. Two F2 populations were developed to identify the candidate genes for HtNB in a humid area with low temperature.

MATERIAL AND METHODS

Plant materials

The single resistant gene HtNB in line P111 was derived from the Indonesian landrace
“Bramadi” by crossing with Tai 183/Zi330. Two F₁ crosses, Huangzao 4 x P111 and B73 x P111 (Huangzao 4 and B73 are susceptible inbreds), were prepared in the spring of 2006, and then self-pollinated to generate two distinct F₂ populations in 2007 on the farm of Huazhong Agriculture University.

Trials were conducted on April 29 in 2008, and on April 25 in 2009. The two F₂ populations contained 291 (Huangzao 4 x P111, F₂, named ZQ1 population) and 356 (B73 x P111, F₂, named ZQ2 population) individuals, respectively. Seeds were grown in plastic trays. After two weeks of germination, the two-leaf seedlings were transplanted into the field. Normal field management was undertaken for the trials.

Pathogen isolation and inoculum production

Race 1 E. turcicum was provided by the Plant Pathology Laboratory of Huazhong Agriculture University. The virulence formula of E. turcicum is Ht2Ht3HtN/Ht1 (Leonard et al., 1989). Inoculum was prepared on potato dextrose agar medium (PDA). This was prepared by boiling 200 g potato for 30 min and then filtering through 4-layer gauze, after which 20 g dextrose and 20 g agar were added and the volume made up to 1 L. The solution was aliquoted into several flasks and sterilized at 121°C for 30 min. A piece of agar (1 x 1 cm) from the maintenance culture medium was seeded on the cooled PDA plate containing 1‰ Amp and cultured (inverted) at room temperature (22°-28°C) for 2-3 weeks in the dark. The piece of agar with greatest amount of spores was transferred onto other plates to generate sufficient spores.

Inoculation and disease evaluation

The parents, F₁ and F₂, and the CK line Zong 3 (highly susceptible) were planted in the field to detect their reaction to E. turcicum race 1. The phenotype identification was conducted at the Tianchi Mountain Research Center of Enshi Autonomous Prefectural Academy of Agricultural Sciences (Tianchi Mountain: 1100 m above sea level., 1118-1900 mm total rainfall., 11.5°-15°C mean annual temperature).

Before application to the plants, conidia were washed with sterile water and adjusted to approximately 10³ conidia/mL water containing 0.1% Tween-20. The conidial suspension was pipetted directly into leaf whorls at the four- to six-leaf stage, usually 300 μL per plant. Three days later, inoculation was performed again to ensure success.

At flowering time, the length and width of each lesion, and its corresponding position according to the top ear were recorded. With regard to no-lesion resistance of HtNB, disease severity was scored according to the following scale: 1 = few and small lesions above the third leaf (below the top ear); 5 = many lesions above and below the third leaf; and 3 = moderate number of lesions below the third leaf.

DNA preparation and genotyping

The genomic DNA from the parents and F₂ individuals was extracted using a CTAB procedure (Zhang et al., 1994). SSR primers for maize chromosome 8 published on the maize genome sequencing platform (http://maizesequence.org) were synthesized by Invitrogen (Shanghai, China).
Thirty-two BAC sequences of 8.06-8.08 bin (ctg362, ctg364, and ctg365) were downloaded and 250 SSR primers derived from the BAC sequences were designed by the online SSR-hunter software (http://www.genome.clemson.edu/contact).

The PCR mixture consisted of 20 ng template DNA, 6 μM primers (forward and reverse primer each), 4 μM dNTP, 2 μL 10X PCR buffer, 37.5 mM Mg²⁺, and 1 U Taq polymerase in a total volume of 20 μL. PCR was performed as follows: 5 min denaturation at 94°C; 32 cycles of 40 s denaturation at 94°C, 45 s annealing at 58°C, and 50 s extension at 72°C; and a final extension for 6 min at 72°C. PCR products were mixed with 10 μL loading buffer and electrophoresed on a 6.7% PAGE gel using 1X TBE buffer.

**Linkage analysis and bioinformatics assay**

Linkage analysis was performed using the MAPMAKER 3.0 software. Distances between markers in centiMorgans (cM) were computed from the Kosambi function (Lander et al., 1987) and the initial logarithm of odds score was 3.0.

Data on the proteins predicted to be encoded by the region between the flanking markers of the gene HtNB was downloaded from the maize sequencing platform (www.maizesequence.org), and analyzed by BLAST (E-value = 10⁻¹⁰) and InterProScan. Several candidate genes related to disease resistance were recorded.

**RESULTS**

**Genetic and phenotypic analysis**

The resistant inbred line P111 only showed few lesions below the third leaf under the ear. In contrast, the susceptible inbred lines, Huangzao4 and B73, had long lesions in the leaves below the third leaf and different amounts of lesions above it. The disease resistance segregation ratio in the ZQ1 F₂ population fitted a three resistant (R) to one susceptible (S) ratio (χ² = 1.4). However, the ratio in the ZQ2 F₂ deviated slightly from 3R:1S (χ² = 4.32) (Table 1). These results are consistent with resistance being conferred by one dominant gene, previously named HtNB in “Bramadi”.

<table>
<thead>
<tr>
<th>Parents, CK line, and progeny population</th>
<th>No. of R and S individuals</th>
<th>Expected ratio</th>
<th>χ²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Huangzao 4</td>
<td>0</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P111</td>
<td>15</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B73</td>
<td>0</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zong 3 (CK)</td>
<td>0</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZQ1 F₂</td>
<td>227</td>
<td>64</td>
<td>3:1</td>
<td>1.4</td>
</tr>
<tr>
<td>ZQ2 F₂</td>
<td>250</td>
<td>106</td>
<td>3:1</td>
<td>4.3</td>
</tr>
</tbody>
</table>

R = resistant; and S = susceptible. Plants with scores of 1 and 3 were considered to be resistant and others with score 5 were regarded susceptible.
Linkage analysis

A total of 61 SSRs on maize chromosome 8 from the public platform MaizeGDB and 250 SSRs from the 32 BACs were used to detect polymorphisms between the parents. Twenty-five SSRs were used to construct the linkage map of ZQ1, while 19 SSR were included in ZQ2. In the ZQ1 population, *HtNB* was located between MAC216826-4 and umc2218 at distances of 3.3 and 3.4 cM, respectively (Figure 1A). In the ZQ2 population, *HtNB* was located between MAC216826-4 (Figure 2) and umc2218 (Figure 3) at distances of 4.3 and 4.0 cM, respectively (Table 2) (Figure 1B). The linkage maps were drawn by MAPDRAWER 3.1.

**Figure 1.** SSR map of *HtNB* on maize chromosome 8. A. Linkage map of *HtNB* using the ZQ1 F2 population. B. Linkage map of *HtNB* using the ZQ2 F2 population. C. The relative location of genes and SSR markers on chromosome 8.

**Figure 2.** Genotyping the flanking SSR marker MK4 of *HtNB* in the ZQ2 population (20 plants are shown). Lane P2 = P111; lane P3 = B73; lanes 1-10 = resistant phenotypes; lanes 11-20 = susceptible phenotypes; lane M = molecular weight marker.
Gene prediction results

The latest assembly of the sequenced maize genome data between umc1384 and umc2218 was downloaded. It includes not only nucleotide data but also amino acid data. The genes had been defined as the entire set of evidence-based genes (predicted by Gramene GeneBuilder) that were then complemented by a set of Fgenesh models. Masked DNA sequence was adopted that did not overlap with the loci of the evidence-based genes. After performing a BLAST search on NCBI, the result showed that 140 hypothetical proteins were included, along with 25 that were annotated as having no function and 60 with no BLAST results. Several of the predicted proteins were speculated to be related to disease resistance.

Several genes in the interval were found to be related to NCLB and may be candidate genes for HtNB. The three most probable proteins are a ubiquitin-like protein, a Ser/Thr kinase protein, and a leaf senescence-like protein. All results are listed in Table 3 and the relative predicted protein location is shown in Figure 4.

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<table>
<thead>
<tr>
<th>Protein</th>
<th>E value</th>
<th>Similarity</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>---NA---</td>
<td></td>
<td></td>
<td>60</td>
</tr>
<tr>
<td>Hypothetical protein</td>
<td></td>
<td></td>
<td>25</td>
</tr>
<tr>
<td>Zinc finger</td>
<td>0</td>
<td>&gt;87.0%</td>
<td>4</td>
</tr>
<tr>
<td>Leucine zipper factor-like</td>
<td>1.2879E-144</td>
<td>83.8%</td>
<td>3</td>
</tr>
<tr>
<td>Ubiquitin-like protein smt3</td>
<td>1.5324E-37</td>
<td>95.3%</td>
<td>4</td>
</tr>
<tr>
<td>Glycine-rich RNA-binding protein 2</td>
<td>2.0124E-63</td>
<td>85.9%</td>
<td>1</td>
</tr>
<tr>
<td>Glycine-rich RNA-binding protein 2</td>
<td>7.6808E-68</td>
<td>86.6%</td>
<td>1</td>
</tr>
<tr>
<td>Leaf senescence protein-like</td>
<td>0</td>
<td>85.8%</td>
<td>2</td>
</tr>
<tr>
<td>Ser/Thr protein kinase</td>
<td>1.0930E-21</td>
<td>60.0%</td>
<td>1</td>
</tr>
<tr>
<td>Adapter-related protein complex 4 epsilon 1 subunit</td>
<td>0</td>
<td>79.4%</td>
<td>1</td>
</tr>
<tr>
<td>Vacuolar proton-inorganic pyrophosphatase</td>
<td>8.5641E-67</td>
<td>95.3%</td>
<td>1</td>
</tr>
<tr>
<td>Phosphoenolpyruvate carboxylase</td>
<td>1.0257E-13</td>
<td>68.0%</td>
<td>1</td>
</tr>
<tr>
<td>bhlh transcription factor</td>
<td>0</td>
<td>80.9%</td>
<td>3</td>
</tr>
<tr>
<td>Retrotransposon</td>
<td>6.3287E-154</td>
<td>57.8%</td>
<td>2</td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td></td>
<td>31</td>
</tr>
</tbody>
</table>

NA = hypothetical protein with no BLAST results.
A single dominant $Ht$ gene targets a specific pathogen race. It suggests that a newly arisen virulent race could escape the resistant gene and cause a susceptible reaction. Hence, pyramiding several $Ht$ genes or integrating $Ht$ genes with R-QTLs (resistant QTLs) is reliable and widely used. To identify the reaction of a single $Ht$ gene, inoculating under controlled conditions is more accurate and could exclude the interference of the environment. In this research, the gene $HtNB$ could delay the appearance of lesions. Therefore, field evaluation was undertaken.

In a previous research, the $HtNB$ gene carrier lines HZ1216 (selected from 77 x Bramadi) and HZ1238 (selected from B37 x Bramadi) were crossed with $HtN$ holder lines, followed by self-pollination to generate the $F_2$. No susceptible phenotype was observed in the $F_2$ populations, and $HtNB$ was predicted to be the same as $HtN$ or its allelic loci or tightly linked loci. As for the relationship between $HtNB$ and $Ht2$, the $F_2$ population and testcross followed a ratio of 12:3:1 and 2:1:1, respectively. In view of this, $HtNB$ and $Ht2$ shared an independent heredity (Xu et al., 1987).

Molecular markers such as RFLP and SSR facilitate the dissection of the genetic foundation of complex traits. Using 99 backcross progeny, the $HtN$ locus was mapped by RFLP markers in the long arm of chromosome 8, following a map order of umc48 - 9 cM-umc30/umc117 - 1.1 cM - $HtN$, and was predicted to be linked to $Ht2$ because of the absence of independent segregation between $HtN$ and $Ht$ (Simcox and Bennetzen, 1993a,b). Further research demonstrated that $Ht2$ is located in the interval of umc48-umc89, near $HtN$ (Zaitlin et al., 1992). $Ht2$ has been fine mapped on 8.05 bin (Yin et al., 2002) and was predicted to be separated from $HtN$ by a distance of 10 cM (Gardiner et al., 1993). Further mapping results for $HtN$ have not been reported.

$HtNB$ was mapped in the same SSR marker interval in both $F_2$ populations. $HtNB$ could be a new gene for NCLB as $HtN$ holds a distinct location in the integrated maize map. QTLs for NCLB in the nearby region have not been reported.
The presence and absence of typical lesions were recorded two weeks after inoculation. Two weeks seems to be a relatively short time for disease extension, and some of the lesion-free individuals showed a resistant phenotype at flowering stage (data not shown). The appearance or non-appearance of typical lesions in two weeks should not be regarded as standard to judge the resistance or susceptibility in the F2 population, because only one, not a family, was tested. Correspondingly, disease resistance evaluation of HtN was conducted in a greenhouse, and the disease reaction was also recorded at two weeks after inoculation. This difference, to some degree, supported the contention that HtNB is distinct from HtN.

In vitro disease evaluation was also performed. P111 showed a small obvious lesion, while typical lesions were observed in the two susceptible lines. The resistant and susceptible lines also showed variable degrees of spore output. Further studies were undertaken to test whether the in vitro evaluation is suitable for disease identification in the F2 population (data not shown).

HtNB, like HtN, delays the appearance of the disease, suggesting a link between the two genes. The fine mapping of HtNB is ongoing and should reveal the difference between them.

In contrast to the HtNB carrier line Bramadi, P111 has a more complex genetic background; therefore, the phenotype in the F2 population may deviate from 3:1 (R:S). P111 is derived from a top cross of Bramadi, Zi330, and Tai183. Zi330 has a quantitative resistance, while Tai183 is a susceptible line. The local race was not virulent for P111, as only few small lesions were seen. In contrast, the two susceptible lines showed long lesions. The lesions of susceptible individuals can extend to entire leaves, especially in the late growing season, and reduce photosynthesis, resulting in a larger yield loss. HtNB extends the period between infection and disease symptom expression. Less-infected leaves on the whole plant, especially the leaves on the top ear, are a prerequisite for the accumulation of light energy.

In the 1980s, race 0 was the dominant race. In this study, the component of the local race was still unknown and it might have affected the reaction to the disease and its appearance. In view of this, members of F3 families were re-tested for disease reaction in 2009. Unfortunately, an unexpected and severe drought ruined this experiment.

The two selected susceptible lines, Huangzao 4 and B73, represent two different levels of disease reaction. B73 is more susceptible than Huangzao 4. There is an obvious difference between ZQ1 and ZQ2. The individuals in ZQ1 showed longer lesions with fewer numbers. In contrast, ZQ2 expressed more lesions of shorter length. This could be explained by the involvement of different QTLs in Huangzao 4 and B73 (data not shown).

Segregation distortion at marker loci was observed in the ZQ2 population. Pollen tube competition, pollen lethals, preferential fertilization, and selective elimination of zygotes have been proven to be related to segregation distortion (Lu et al., 2002). Molecular marker analysis facilitates the identification of chromosomal regions consistently associated with segregation distortion. The region among markers A1065-umc7-gst1 in chromosome 8.07-8.08 bin was regarded to be a candidate segregation distortion region (SDR). The segregation of this region favored the genotype from the parent B73 (Lu et al., 2002). In the ZQ2 population, a larger contribution of the genotype obtained from B73 is observed in this region, indicating that some gametophytic factor associated with segregation distortion was involved in B73. This could explain why susceptible individuals appearing in ZQ2 were more than expected. In addition, several studies suggested that the deficiency of nitrogen fertilizer may increase the severity of the disease (Pan et al., 1980). Normal field management may promote the development of the disease, especially in the heading stage.
Gene mapping of HtNB

Plant disease resistance includes basal defense and resistant (R) gene-mediated immune response. The guard model explains how the R genes’ products recognize the avirulent (Avr) proteins in the pathogen (Belkhadir et al., 2004). Recently, several cases of effector perception support a new model named the Decoy Model, in which duplication of the effector target gene or independent evolution of a target mimic could relax evolutionary constraints and result in a decoy that could be solely involved in effector perception (van der Hoorn and Kamoun, 2008). Up to 2004, 51 R genes were cloned in plants, most of which display the typical LRR (leucine-rich repeat) structure of the NBS-LRR gene (Wang et al., 2004). Homology-based techniques and bioinformatics were used to predict the RGA (resistant gene analog) in the whole maize genome, in which RGAs in chromosome 8.07-8.08 bin were detected (Xiao et al., 2007).

Ubiquitination may play an important role in plant disease resistance. For example, XA21 is a Ser/Thr kinase protein in rice (Oryza sativa) conferring disease resistance. XA21-binding protein 3 (XB 3) is an ubiquitin ligase. Coimmunoprecipitation assays have shown that XB3 is a substrate for XA21. Transgenic results showed that a reduced level of XB3 led to a decreased level of XA21 protein expression (Wang et al., 2006). Glycine-rich protein (GRP) consists of two different types of protein. Some GRPs with a typical eukaryotic N-terminal signal peptide have been found to be important structural proteins. Other GRPs contain a common RNA-binding domain consisting of 80-100 amino acids and may be located in the nucleus. Several factors could influence the expression of GRP, such as mechanical damage and fungal infection (Chen et al., 2005). In addition, Me-JA and ethylene are known to regulate the expression of GRPs (Molina et al., 1997). In the HtNB region, a hypothetical protein was identified that is predicted to be involved in plant senescence and shares a 85.8% amino acid similarity with leaf senescence protein-like in rice (E = 2e^-62). A predicted Ser/Thr kinase protein was also found in the region.

As the candidate gene prediction of the target region was conducted with B73 BAC sequence, some deleted fragments in the B73 genome were disclosed. For further research, a BAC library of P111 is required, followed by sequencing of the positive clones and exploration of candidate genes.

This is the first mapping result for HtNB and will facilitate further fine mapping. A larger F2 population will be prepared and the susceptible individuals could be regarded as a group for identifying re-exchange between the resistant gene and molecular markers. Gene pyramiding with conventional methods is very difficult for epistatic effects and residual effects, so marker-assisted selection will be used as an aid for early selection. The fine-mapping and cloning of resistant genes will help in creating better varieties that can adapt to a variety of adverse conditions.

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Gene mapping of HtNB


