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RESEARCH ARTICLE

Marker-assisted introgression of turcicum leaf blight (*Ht1*) resistant gene into northeastern Himalayan region maize landrace

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Abstract

The local people of north-eastern Himalayan region (NEHR) cultivate the maize landraces and consume them as food. These landraces possess desirable agronomic traits but are susceptible to turcicum leaf blight (TLB) disease caused by the fungus *Exserohilum turcicum*. Thus, we aimed to screen maize landraces under field conditions and introgress the *Ht1* gene into the susceptible landrace. Screening of four landraces against TLB was conducted under artificially inoculated field conditions during *Kharif* 2023, with two standard checks. Disease reaction rating on a scale of 1-9 was used to calculate the percent disease index (PDI) values of landraces. Among the four landraces, two - LMC -15 and LMC-16 - were identified as susceptible, with a disease rating of 8, while LMC-4 and LMC-7 received a rating of 6 and were categorized as moderately susceptible. The F_1 generation was produced during the spring season of 2024 through a cross between the donor parent BML-6 and the susceptible recurrent parent LMC-15. The true F_1 plants were evaluated using a SSR marker *umc1042*, which is linked to the TLB resistance gene *Ht1*. Genotyping of 120 F_2 plants was performed using the *umc1042* SSR marker. This marker exhibited a segregation ratio of 27:61:32 in the F_2 population. The chi-square value for the genotype was 0.45, which is below χ^2 ($p \leq 0.05$) and therefore considered non-significant, indicating a good fit to the expected 1:2:1 ratio. Overall, the findings confirm that MAS is an efficient and reliable approach for introgressing *Ht1* into susceptible maize lines.

Keywords: *Exserohilum turcicum*; *Ht1* gene; maize; marker-assisted selection; NEHR; SSR marker

Introduction

Maize is the second most important crop in the north-eastern Himalayan region (NEHR) of India, following rice and is predominantly cultivated in rainfed upland areas (1). The Indian NEHR is the centre of maize diversity, with a unique collection of landraces. These landraces have valuable agronomic traits and possess the ability to tolerate stressful conditions; therefore, they are carefully preserved and traditionally maintained by NEHR farmers (2). Over the last few decades, turcicum leaf blight (TLB) has severely affected maize production, causing significant yield losses (3). TLB, caused by the fungus *Exserohilum turcicum*, is a destructive foliar disease of maize (4). The disease is found worldwide, mainly in regions where the relative humidity ranges from 75 % to 90 % and temperature ranges from 22 °C to 25 °C during the growing season (5, 6). In India, the disease occurs most frequently across all major maize-growing areas during both the rainy (*Kharif*) and winter (*Rabi*) seasons (7). Symptoms initially appear as small, oval, gray-green, water-soaked spots that later develop into elongated, spindle-shaped, necrotic lesions (8).

Utilizing resistant cultivars is the most effective, economical and environmentally friendly strategy for managing TLB. Several qualitative TLB-resistance *Ht* genes have been identified, namely *Ht1*, *Ht2*, *Ht3* and *HtN* corresponding to their locus designations (9, 10). The *Ht1* gene has been widely studied and used in maize breeding for TLB resistance because it was the first major gene identified for disease and demonstrated durable and consistent effects across environments (11). It produces a chlorotic-lesion resistance phenotype rather than typical necrotic lesions; in resistant plants, lesions remain chlorotic with limited sporulation, thereby reducing disease spread (12).

Marker-assisted selection (MAS) is considered a powerful and efficient breeding strategy because it enables rapid and accurate selection of target genes and its use has produced notable improvements in maize breeding programs aimed at developing TLB-resistant cultivars (13, 14). However, limited information is available on *Ht* genes and associated molecular markers for use in MAS in breeding maize for TLB resistance. Thus, the present study was undertaken to identify resistant (R) and susceptible (S) NEHR maize landraces through artificial inoculating with *E. turcicum* and to introgress the TLB-resistant gene *HT1* into a susceptible NEHR maize landrace.

Materials and Methods

Plant materials and development of mapping population

Four agronomically superior, high-yielding and popular landrace were collected from different district of Manipur. The landraces tested were LMC-4, LMC-7, LMC-15, LMC-16 and the checks used were VLB-55 (resistant check) and early composite (susceptible check). They were inoculated with *E. turcicum* to determine their resistance to TLB, using a rating score of 1-9. The seeds of NEHR landraces were obtained from Central Agricultural University, Imphal, India and used in this study. Another genotype BML-6 (TLB resistant), was used as the donor parent and was provided by ICAR-IIMR, Ludhiana. LMC-15, which exhibiting the highest PDI value (susceptible to TLB), was utilized as the recurrent parent to introduce the *Ht1* gene from the donor parent BML-6.

The study involved 120 F_2 plants, produced by crossing LMC-15 and BML-6 in the spring season of 2024 at the research farm of the Department of Genetics and Plant Breeding at CAU, Imphal. This farm is situated at a latitude of 24°82'N and a longitude of 93°90'E, at an elevation of 790 m MSL in the Imphal West district of Manipur. The hybrid seeds from this cross were collected separately and planted in the *Kharif* season of 2024 along with both parents. The hybridity of F_1s was validated using *Ht1* gene linked *umc1042* SSR marker and the true F_1s were self-pollinated to produce the F_2 generation.

Inoculation and disease evaluation

Mass multiplication of inoculums

Pure culture media of *E. turcicum* obtained from the Department of Plant Pathology, Central Agricultural University, Imphal. Mass multiplication of *E. turcicum* was carried out on sterilized whole sorghum grains (15). A layer of sorghum grain, approximately one inch in depth, is placed in a conical flask and soaked in water for duration of 4-6 hr. After soaking, excess water was removed. The flasks containing sorghum grains were autoclaved twice on alternate days, seeded with fungus under aseptic conditions and incubated at 25-27 °C. Every 2-3 days, the flasks were shaken to promote uniform growth of *E. turcicum* on sorghum grains. After an incubation period of approximately two weeks, the material was ready for inoculation (Fig. 1).

Artificial inoculation

Suspensions of *E. turcicum* spores were quantified using a haemocytometer and maintained a concentration of 3×10^5 spores mL^{-1}. The diluted suspension was used for inoculating plants as a spray applied using a knapsack sprayer directed toward the whorl. Inoculations were preferably done late in the afternoon. Artificial inoculation was performed twice, at 30 and 40 days after sowing (Fig. 1).

Disease score

The percent disease index (PDI) of TLB was calculated at four stages, 40, 50, 60 and 70 days after sowing and the disease reaction was measured using a 1-9 scale (4) (Table 1). The PDI was calculated using the formula (16).

a) Pre-soaking of sorghum grains **b) TLB pure culture** **c) Sorghum grains seeded with fungus**

d) Complete mycelia growth on sorghum grains **e) Spraying of *E. turcicum***

Fig. 1. Mass multiplication of *E. turcicum* on sterilized sorghum grains and artificial inoculation of pathogen.

Table 1. Rating scale for TLB disease in maize

Rating scale	Degree of infection (percent DLA*)	PDI**	Disease reaction
1.0	Nil to very slight infection (≤ 10%)	≤11.11	Resistant (R) (score: ≤ 3.0) (PDI: ≤ 33.33)
2.0	Slight infection, a few lesions scattered on two lower leaves (10.1-20 %)	22.22	
3.0	Light infection, moderate number of lesions scattered on four lower leaves (20.1-30 %)	33.33	
4.0	Light infection, moderate number of lesions scattered on lower leaves, a few lesions scattered on middle leaves below the cob (30.1-40 %)	44.44	Moderately resistant (MR) (Score: 3.1-5.0) (PDI: 33.34-55.55)4
5.0	Moderate infection, abundant number of lesions scattered on lower leaves, moderate number of lesions scattered on middle leaves below the cob (40.1-50 %)	55.55	
6.0	Heavy infection, abundant number of lesions scattered on lower leaves, moderate infection on middle leaves and a few lesions on two leaves above the cob (50.1-60 %)	66.66	Moderately susceptible (MS) (score: 5.1-7.0) (PDI: 55.56-77.77)
7.0	Heavy infection, abundant number of lesions scattered on lower and middle leaves and moderate number of lesions on two to four leaves above the cob (60.1-70 %)	77.77	
8.0	Very heavy infection, lesions abundant scattered on lower and middle leaves and spreading up to the flag leaf (70.1-80 %)	88.88	Susceptible (S) (score: > 7.0) (PDI: > 77.77
9.0	Very heavy infection, lesions abundant scattered on almost all the leaves, plant prematurely dried and killed (>80 %)	99.99	

*DLA: diseased leaf area; **percent disease index (PDI).

$$PDI = \frac{\text{Sum of individual disease score}}{\text{Total number of plants scored x Maximum disease score}} \times 100$$

Area under disease progress curve (AUDPC) analysis

The disease progress curve consisted of disease severity (%) recorded at 40, 50, 60 and 70 days after sowing, a 10-day intervals starting from the onset symptom. To ensure consistent field evaluation, the disease progress curve was prepared from PDI readings. AUDPC is used to quantify the epidemic onset and the time required for the blight to reach its peak. The derived disease parameter, AUDPC, was calculated using the equation (17).

$$AUDPC = \sum_{i-1}^{n-1} \frac{Y_i + Y_{i+1}}{2} \times (t_{i+1} - t_i)$$

Where, Y_i = disease at the i^{th} observation, T_i = time (days) at the i^{th} observation and n = total number of observations.

Molecular marker analysis

Genomic DNA was extracted from three-week-old seedling using the modified CTAB method (18). The quality and quantity of extracted DNA was examined using 0.8 % agarose gel electrophoresis and a Nanodrop spectrophotometer and samples were diluted to a final concentration of 90 ng/µL for PCR amplification. The F_1 hybridity test was performed with the *umc1042* gene-linked marker.

PCR amplification was carried out in a 10 µL reaction mixture containing 1 µL of 90 ng/µL gDNA, 0.5 µL each of forward and reverse primers, 5 µL of 2× Takara green PCR Master Mix and 3 µL of nuclease -free water, in 96-well PCR plates sealed with a thermal seal in a QIAmplifier 96 thermal cycler. The amplification profile consisted of an initial denaturation at 95 °C for 5 min, followed by 35 cycles: denaturing at 95 °C for 45 sec, annealing at 58 °C for 45 sec, extension at 72 °C for 45 sec and a final extension at 72 °C for 8 min.

The amplified PCR products were run at on 4 % agarose gel using 1× TAE buffer at 5 V/cm for 3 hr and visualized on a UV transilluminator. PCR product sizes were determined using a 100 bp DNA ladder.

Genotyping of F_2 population

The presence of the TLB resistance gene in all F_2 plants was evaluated using the gene-linked molecular marker *umc1042*. Amplicons were classified as "A" for the male parent (homozygous dominant), "B" for the female parent (homozygous recessive) and "H" for the heterozygous individuals.

Statistical analysis

A chi-square test was used to evaluate the segregation distortion of the SSR marker from the expected Mendelian 1:2:1 ratio in the F_2 population. This test was also utilized to assess marker allele frequency, homogeneity and the distribution of marker genotypes in order to investigate the causes of segregation distortion. The chi-square value is determined using the formula provided.

$$\chi^2 = \sum \frac{(O - E)^2}{E}$$

Where, E = expected value and O = observed value.

Results

Field evaluation of maize landraces against TLB

The evaluation of resistance to TLB in NEHR maize landraces was conducted during the *Kharif* season of 2023. The results showed clear and distinct responses of the landraces to the TLB pathogen under artificially inoculated conditions. Four widely recognized NEHR maize landraces, LMC-4, LMC-7, LMC-15 and LMC-16, along with two standard checks-VLB-55 (resistant check) and early composite (susceptible check)-were inoculated with *E. turcicum* to evaluate their resistance to TLB.

The mean disease scores at four different growth stages of the plants were recorded and the PDI for each stage was calculated. The AUDPC values were derived from these four PDI values (Table 2). The graphs were prepared using AUDPC values (Fig. 2).

The experiment showed clear variation in disease response among the landraces. Among four landraces, two were identified as susceptible: LMC-15 (PDI 82.22 % and LMC-16 (PDI 80 %) with a disease rating of 8 (Fig. 3). In contrast, LMC-4 (PDI 71.11 %) and LMC-7 (PDI 66.66 %) received a rating of 6 and were categorized as moderately susceptible.

Table 2. Presenting AUDPC values for TLB of maize landraces and checks, derived from PDI

Sl. No.	Landraces/Genotypes	PDI (%)	AUDPC	Disease reaction
1.	LMC-4	40th DAS: 22.22 50th DAS: 44.44 60th DAS: 64.44 70th DAS: **71.11**	1666.55	Moderately susceptible
2.	LMC-7	40th DAS: 17.77 50th DAS: 42.22 60th DAS: 46.66 70th DAS: **66.66**	1399.8	Moderately susceptible
3.	LMC-15	40th DAS: 40.00 50th DAS: 57.77 60th DAS: 71.11 70th DAS: **82.22**	2099.9	Susceptible
4.	LMC-16	40th DAS: 37.77 50th DAS: 53.33 60th DAS: 68.88 70th DAS: **80.00**	1999.8	Susceptible
5.	Early composite (susceptible check)	40th DAS: 42.22 50th DAS: 64.44 60th DAS: 73.33 70th DAS: **84.44**	2222.1	Susceptible
6.	VLB-55 (resistant check)	40th DAS: 11.11 50th DAS: 15.55 60th DAS: 20.00 70th DAS: **26.66**	599.9	Resistance

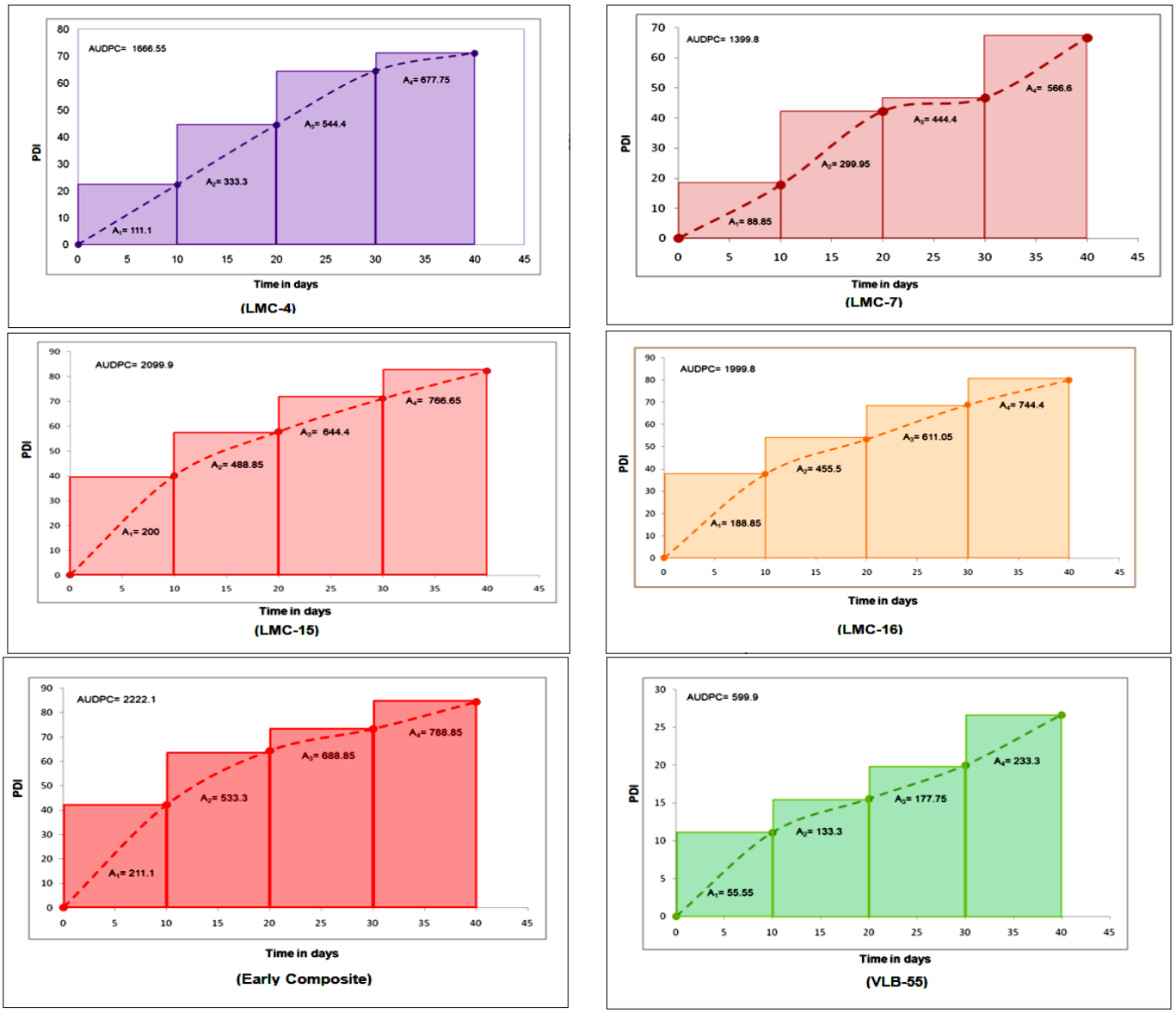

Fig. 2. Graphical representation of disease reaction (AUDPC).

1. LMC - 15

2. Susceptible check (Early composite)

3. Resistant check (VLB - 55)

Fig. 3. Disease reaction of LMC-15 with resistant and susceptible checks.

Introgression of *Ht1* resistance gene against TLB into the maize landrace

This study was conducted with the aim of introducing the *Ht1* resistance gene for Turcicum Leaf Blight into susceptible maize landrace. In the *spring* season of 2024, the recurrent parent LMC-15 (female) was crossed with the donor parent BML-6 (male parent), which carries the *Ht1* gene known for TLB resistance. After confirming the F_1's for the existence of target gene obtained from the crosses, the true F_1's were raised and self-pollinated (Fig. 4).

Validation of gene linked molecular markers between parents

Before starting the crossing program, the disease resistance of the parents was confirmed. In this study, both the donor and recipient parents were raised during the *spring* season of 2024 and assessed for the presence of specific resistance genes using previously identified molecular markers (Table 3). Donor and recipient parents were distinguished using polymorphic SSR markers, specifically gene-linked markers such as *umc1042*, *bnlg1721*, *bnlg198* and *bnlg1335*. The umc1042 marker exhibited polymorphism between the recurrent and donor parents, while the other markers revealed monomorphism between the parents for the *Ht1* gene. The *umc1042* marker associated with the *Ht1* gene was amplified at 110 bp in the donor (BML-6) and a 100 bp fragment in recurrent parent LMC-15 (Fig. 5).

The molecular screening results for the target gene in the parental lines indicated that the gene-based marker effectively differentiated resistant lines from susceptible ones in a co-dominant manner, enabling the identification of homozygous and heterozygous states. As a result, plants carrying the gene in the F_2 generations could be accurately identified without false positives.

Generation and confirmation of F_1's

From the cross between LMC-15 and BML-6, a total of 15 F_1 plants were generated and raised during the *Kharif* season of 2024. Leaf samples were collected at the seedling stage, DNA was isolated and each and each plant was tagged. PCR was conducted using the gene -linked marker *umc1042*, which showed polymorphism between the donor and recurrent parents.

After genotyping, 12 plants showed heterozygosity, confirming them as true F_1s, while the remaining plants showed recurrent parent bands, indicating that they were selfed. The presence of single bands revealed that those plants were not true hybrids, as SSR primers are co-dominant (Fig. 6).

Generation of F_2 population and genotyping of F_2 population for TLB resistant genes

The true F_1 plants with the TLB resistant gene were self-pollinated to produce the F_2 generation at the research farm of the Department of

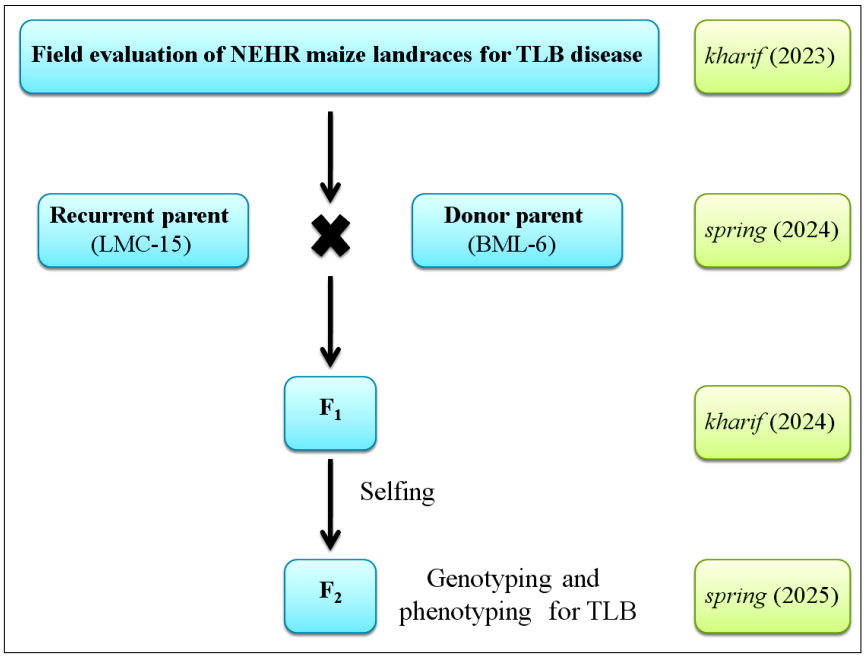

Fig. 4. Schematic representation for the development of TLB disease resistance lines using phenotypic and genotypic selection.

Table 3. Information about the molecular markers utilized for foreground selection related to the gene responsible for TLB resistance

Sl. No.	Marker	Gene	Chr. No.	References
1	*umc1042*	*Ht1*	2	(3)
2	*bnlg1721*	*Ht1*	2	(3)
3	*bnlg198*	*Ht1*	2	(28)
4	*bnlg1335*	*Ht1*	2	(28, 29)

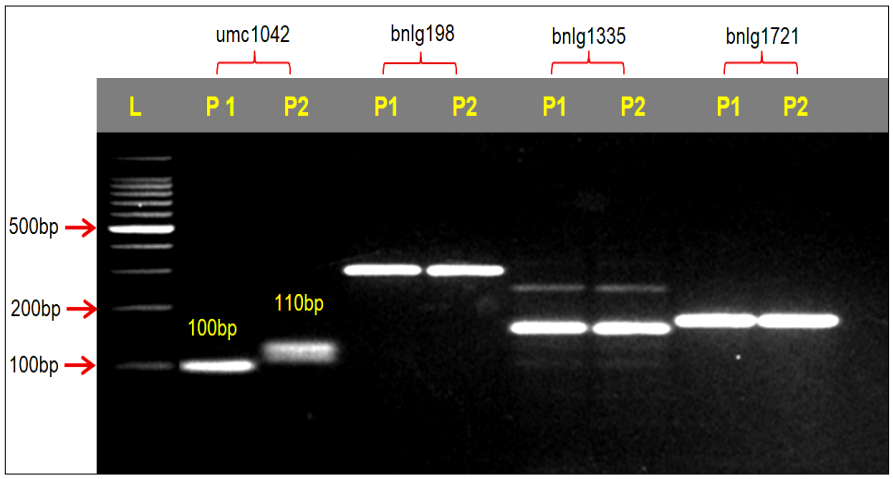

Fig. 5. Validation of gene linked molecular markers between parents. L: 100 bp ladder; P1: recurrent parent (LMC-15); P2: donor parent (BML-6).

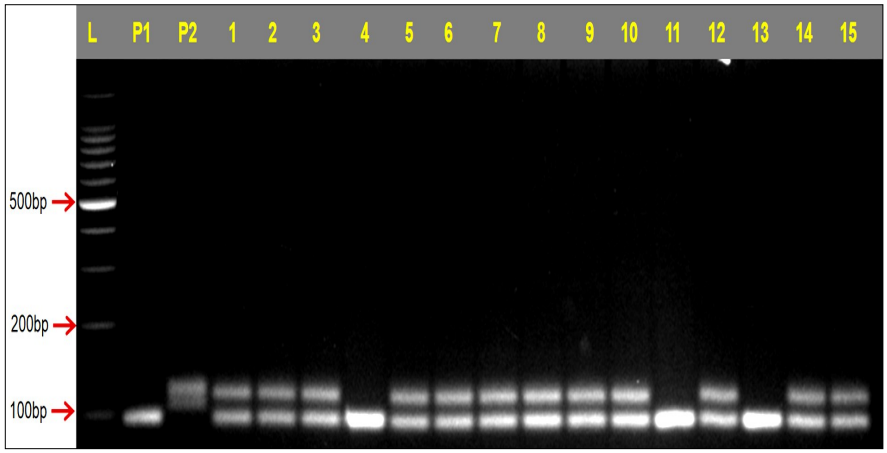

Fig. 6. Hybridity confirmation of the F_1's for *Ht1* gene using *umc1042* marker. L: 100 bp ladder; P1: recurrent parent (LMC-15); P2: donor parent (BML-6); 1-15: F_1's.

Table 4. Segregation pattern of *umc1042* maker in F_2 population and their goodness of fit of genotypic ratio

Total number of F_2 plants	Segregation pattern						Expected ratio		
	Observed			Expected					
	A	H	B	A	H	B	A:H:B	X^2	*p*-value
120	27	61	32	30	60	30	1:2:1	0.45	5.99

A: homozygous dominant; H: heterozygotes; B: homozygous recessive; significant value of *p* at 0.05 is 5.99.

Genetics and Plant Breeding, CAU, Imphal, during the *Kharif* of 2024. Genotyping of 120 F_2 plants was done using the *umc1042* SSR marker, which showed polymorphism between LMC-15 and BML-6. The marker *umc1042* exhibited a segregation ratio of 27:61:32 (homozygous dominant: heterozygotes: homozygous recessive) in the F_2 population. The findings indicate that the chi-square value for the genotype was 0.45, which is below X^2 ($p \leq 0.05$) and thus considered non-significant. Therefore, the chi-square test demonstrated a good fitness for the 1:2:1 ratio (Table 4). Due to the study's aim of achieving durable and extensive resistance, only the 27 plants that possess the target gene in a homozygous state were selected in the field for additional assessment of their yield and agronomic characteristics (Fig. 7).

Phenotyping of F_2 mapping population

A total of 120 F_2 plants were evaluated for TLB disease under field conditions. Among them, 12 plants showed resistance reactions, 76 plants exhibited moderate resistance, while 27 plants showed moderate susceptible and 5 plants showed susceptibility. This distribution resulted in a phenotypic ratio of 3R:1S (88 resistance:32 susceptible), suggesting that TLB resistance is governed by a dominant gene (Table 5).

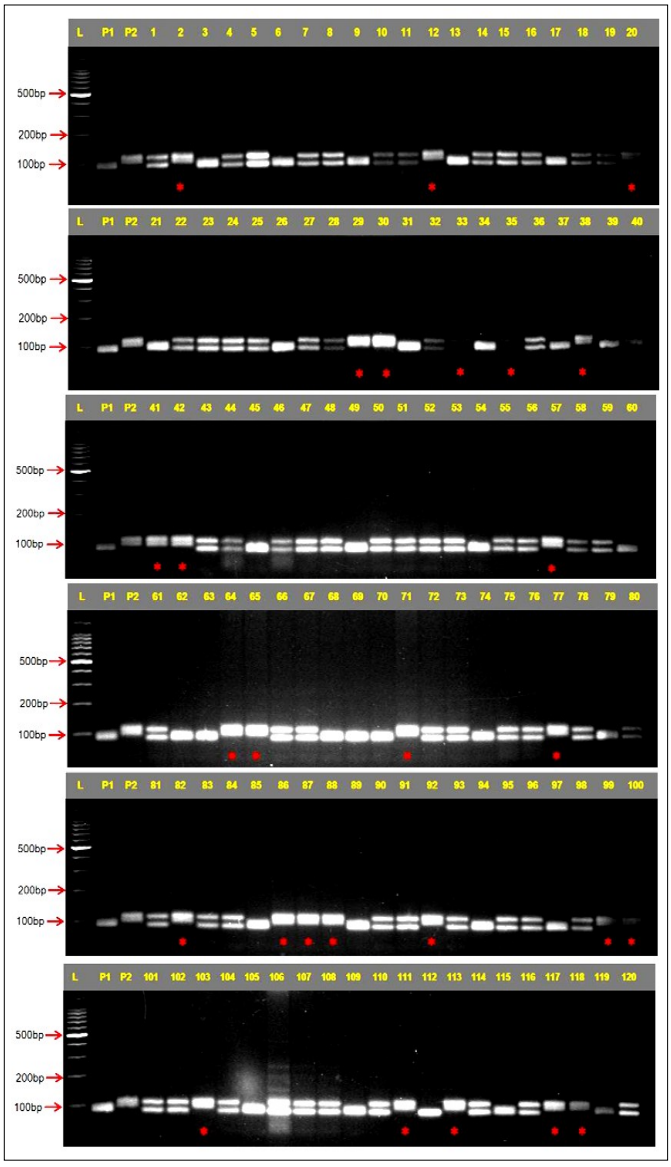

Fig. 7. Foreground selection in F_2 plants for the *Ht1* gene, along with the parental lines, utilizing the *umc1042* marker. L: 100 bp ladder; P1: recurrent parent (LMC-15); P2: donor parent (BML-6); F_2: 1-120.

Discussion

Field screening of maize under natural epiphytotic conditions revealed significant variation in resistance to TLB caused by *E. turcicum*. The field conditions in Imphal, characterized by high relative humidity and moderate temperatures during the crop growth period**,** were favourable for the development of TLB. The differential host response observed in this study indicate the presence of moderately susceptible and susceptible landraces in the tested materials. Such variability has also been reported in other maize-growing regions of India and abroad, underscoring the availability of valuable resistance sources for breeding (19, 20). The present research found that LMC-15 and LMC-16 were susceptible, while LMC-4 and LMC-7, were classified as moderately susceptible (21-23).

The present study demonstrates the successful introgression of the *Ht1* gene**,** which confers resistance to TLB caused by *E. turcicum*, into a susceptible maize background and its validation in the F_2 segregating population through MAS. The SSR marker umc1042 demonstrated polymorphism between the two parental lines, amplifying a 110 bp allele in the resistant parent and 100 bp allele in the susceptible parent. Chi-square analysis of the genotypic data from 120 F_2 plants amplified using the *umc1042* marker showed a non-significant deviation, indicating a good fit to the expected 1:2:1 segregation ratio.

Table 5. Segregation pattern of individual F_2 population regarding TLB disease resistance and their goodness of fit of phenotypic ratio

Total number of F_2 plants	Reaction to TLB disease				Expected ratio		
	Observed		Expected				
	R	S	R	S	R:S	X^2	*p*-value
120	88	32	90	30	3:1	0.17	3.84

R: number of resistance plants; S: number of susceptible plants; significant value of *p* at 0.05 is 3.84.

The segregation pattern in the F_2 generation revealed the expected distribution of resistant and susceptible individuals, consistent with the monogenic inheritance model (24, 25). These results confirm that *Ht1* was effectively introgressed and expressed in the target genetic background. MAS ensure precise and early detection of resistant individuals, thereby accelerating the breeding cycle and reducing the cost and time associated with multiple generations of field testing. Similar benefits of MAS in the selection of disease resistance genes in maize have been reported and validated in subsequent studies (26, 27). Overall, the findings confirm that MAS is an efficient and reliable approach for introgressing *Ht1* into susceptible maize lines. The F_2 segregating population demonstrated clear evidence of resistance, validating both the effectiveness of *Ht1* and the utility of marker-based selection. Moving forward, integrating MAS with conventional backcrossing, pyramiding *Ht1* with other resistance sources and monitoring pathogen variability will be essential for developing durable, high-yielding, TLB-resistant maize hybrids.

Conclusion

The study focused on improving maize landraces from the NEHR of India that are highly valued by farmers but remain susceptible to TLB. Four landraces were screened under artificial inoculation and two (LMC-15 and LMC-16) were identified as highly susceptible, while the other two showed moderate susceptibility. To introduce resistance, the *Ht1* gene from a resistant donor parent (BML-6) was crossed into the susceptible landrace LMC-15. The resulting F1 plants were validated using a co-dominant SSR marker (*umc1042*), confirming the presence of the target gene. The integration of phenotypic evaluation with marker-assisted selection proved to be an effective approach for incorporating resistance genes. This study successfully demonstrated the introgression of the TLB-resistant gene *Ht1* into a NEHR maize landrace. Moreover, the developed material can be advanced using the single seed descent (SSD) method to create recombinant inbred lines (RILs) for further genetic analysis and breeding.

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Authors' contributions

RSC conducted the complete research process, including drafting and preparing the initial version. PK supplied the resources, oversaw the study, and revised the manuscript. TRD, PS, NN, BS, SK and VC were involved in the research methodology, manuscript creation and editing. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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