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RESEARCH ARTICLE

Marker-assisted elimination of necrosis gene (*Ne1*) present in wheat varieties HD2967 and HD2733

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Abstract

Hybrid necrosis in wheat occurs because of two dominant complementary genes, *Ne1* and *Ne2,* located on chromosomes 5BL and 2BS, respectively. While transferring rust resistance genes in some Indian wheat varieties, hybrid necrosis was observed with HD2967 and HD2733, while no necrosis was observed with HD2932 and HD3059. A marker-assisted backcrossing program was initiated to eliminate the necrosis gene present in HD2967 and HD2733. Markers linked to *Ne1* (*Xbarc74*) and *Ne2* (*Leq54_LrLC10* and *Leq22_LrLC10*) genes were used in marker validation using a set of genotypes. The linked markers showed the presence of the *Ne1* gene in wheat varieties HD2967, HD2733 and positive check of *Ne1*, C306 and *Ne2* gene in Parula as that of positive check Sonalika. The wheat varieties HD2932 and HD3059 showed the absence of *Ne1* and *Ne2* genes as negative checks, Agra Local and NI5439. For marker-assisted elimination of *Ne1* from HD2967 and HD2733 or substitution of dominant *Ne1* allele with recessive *ne1* allele, HD2932 was used as a donor parent. Near isogenic Lines (NILs) of HD2967 (RPG = 96.80%) and HD2733 (RPG = 95.55%) were identified in BC_2F_3 generations. Plants homozygous for recessive allele (*ne1ne1*) and dominant allele (*Ne1Ne1*) were crossed with *Ne2* carrier Sonalika (*Ne2Ne2*) to test the effectiveness of marker-assisted selection. While all the F_1s of *Ne1Ne1* died at the seedling stage, the F_1s of *ne1ne1* were viable and produced normal seeds. The present study validated the available molecular markers of *Ne1* and *Ne2* and used these markers to develop NILs devoid of necrosis gene *Ne1*. The superior genotypes (NILs) without any necrosis gene can be used freely in developing superior male sterile (A) lines for hybrid breeding programmes or in the transfer of genes of economic importance without fear of getting hybrid necrosis.

Keywords: Wheat, necrosis gene, *Ne1*, *Ne2*, marker validation, marker assisted elimination

Introduction

The F_1 hybrids from many inter-varietal, inter-specific, and intergeneric crosses in wheat often show deleterious traits such as hybrid necrosis, hybrid chlorosis, hybrid dwarfism, and apical lethality (Tomar et al. 2007). These deleterious phenomena are often caused by genetic interactions leading to premature and gradual death of certain F_1 hybrids (Hermsen 1966). Hybrid necrosis manifests itself as the gradual and premature drying of leaves in F_1 hybrids, leading to the premature death of plants. Hybrid necrosis appears once the seedling is two leaves old. The drying or necrosis first appears at the tip of the oldest leaf, and it progresses gradually to include the entire first, second, and third leaf, as well as an abortive fourth leaf, ultimately leading to the death of the seedling (Caldwell et al. 1943). Besides wheat, hybrid necrosis has been documented in several other crops, e.g., barley (Wiebe 1934), *Arabidopsis thaliana* L. (Smith et al. 2011; Muralidharan et al. 2014; Świadek et al. 2017), tobacco (*Nicotiana tabacum* L.) (Tezuka and Marubashi2006**;** Tezuka 2012; Liu and Marubashi 2014) and rice (Ichitani et al. 2007;

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Saito et al. 2007). In most of the reported cases, hybrid necrosis was found to be controlled by interaction of two non-allelic complementary genes, such as *Ne1–Ne2* in wheat (Hermsen 1963; Zeven 1972), *Hwa1–Hwa2*, *Hwc1–Hwc2*, and *Hwi1–Hwi2* in rice (Ichitani et al. 2007; Kuboyama et al. 2009; Chen et al. 2014), *Le3–Le4* in cotton (Song et al. 2009), *Rcr3/Ne–Cf-2* in inter-specifc tomato hybrids (Krüger et al. 2002) and *DM1–DM2* and *SRF3–RPP1* in Arabidopsis (Bomblies et al. 2007; Alcázar et al. 2010). Tomar et al. (1988) reported hybrid necrosis in several inter-specific crosses involving *Triticum aestivum* with *T. durum, T. turanicum, T. polonicum* and *T. pyramidale*. Earlier Nishikawa (1967) and Zeven (1976) also reported hybrid necrosis in inter-specific crosses.

The genetic basis of hybrid necrosis in wheat is a complex yet fascinating area of study. The complementary genes causing necrosis in wheat, namely *Ne1* and *Ne2,* were found to be located on chromosome arms 5BL and 2BS, respectively (Tsunewaki 1961; Hermsen 1966; Zeven 1972; Nishikawa et al. 1974). The variation in the degree of necrosis among different F_1s was attributed to multiple alleles of *Ne1* and *Ne2* (Hermsen 1963). These alleles were characterized as strong(s), moderate(m), weak(w), and with intermediate strengths (mw and ms). The presence of multiple alleles at each locus of *Ne1* and *Ne2* leads to different combinations of *Ne* alleles, resulting in varying degrees of necrosis in different crosses (Vikas et al. 2013). Chu et al. (2006) mapped necrosis genes, *Ne1* and *Ne2*, with microsatellite markers, *Xbarc74* and *Xbarc55,* on chromosomes 5BL and 2BS at a genetic distance of 2.2 and 2.3 cM, respectively. Fine mapping of the necrosis gene *Ne1* identified three co-segregating markers, *Xwgrc3146*, *Xwgrc3147* and *Xwgrc3150* (Li et al. 2021). Later in the same year, Si et al. (2021) used map-based cloning to characterize the necrosis gene *Ne2*, which was found to encode a coiled coil-nucleotide binding site-Leucine-Rich Repeat (CC-NBS-LRR) protein.

The distribution of *Ne1* and *Ne2* in wheat varieties has significant implications for wheat breeding programs. A study on the distribution of *Ne1* and *Ne2* revealed a predominance of *Ne2* in Indian wheat varieties compared to *Ne1* (Vikas et al. 2013). While *Ne1* is of Indian origin and common in landraces and old varieties, the predominance of *Ne2* occurred after the introduction of semi-dwarf Mexican wheat varieties, which are mostly *Ne2* carriers, and also due to the extensive and continuous use of germplasm from Mexican and European origin in the hybridization program (Vikas et al. 2013). Indian wheat varieties HD2967 and HD2733 are high-yielding varieties released more than a decade ago for cultivation in different zones of the country. When used in the crossing program, these varieties showed hybrid necrosis in several crosses. Since HD2967 and HD2733 carry the *Ne1* allele, their use in wheat breeding programs is restricted. Eliminating the necrosis gene *Ne1* in these superior genotypes will enable breeders to use them in hybrid or gene transfer programs without encountering hybrid necrosis. Therefore, the present study was conducted to eliminate the necrosis gene(s) by substituting the *Ne1* allele with the *ne1* allele in HD2967 and HD2733.

Materials and methods

Plant materials

The plant material comprised bread wheat genotypes Agra Local (AL), NI5439, HD2967, HD2733, HD2932, HD3059, Sonalika, C306 and Parula. Wheat cultivars C306 and Sonalika were used as positive checks for necrosis alleles *Ne1* and *Ne2,* respectively. Agra Local and NI5439 were used as negative checks as they have been extensively used in different crossing programmes without manifestation of necrosis. Varieties HD2967, HD2733, HD2932, HD3059, and a Mexican wheat genotype Parula (with which HD2967 and HD2733 produced hybrid necrosis) were used as test genotypes. HD2967 and HD2733 were used as recurrent parents (RP) in the marker-assisted backcross breeding programme, while HD2932 (*ne1ne1ne2ne2*) was used as donor parent (DP) for the *ne1* allele.

Molecular markers used in marker assisted selection

SSR marker *Xbarc74* was used in marker-assisted selection of necrosis gene *Ne1*. For *Ne2* two indel-specific markers, Leq54_LrLC10 and Leq22_LrLC10 were used in the current study. The markers *Xbarc74, Leq54_LrLC10,*and *Leq22_LrLC10* are co-dominant markers and can differentiate heterozygotes from homozygotes. The details of markers used in the current study are provided in Table 1. After validation, the linked molecular marker was used in foreground selection to identify plants carrying recessive allele (*ne1*) either in heterozygous or homozygous state. Parental polymorphism between RPs HD2967 and HD2733 and DP HD2932 was carried out with 642 SSR primers covering 21 wheat chromosomes. Polymorphic markers were used in background selection in each backcross generation.

Marker assisted backcrossing to substitute the dominant allele Ne1 with the recessive allele ne1

The recurrent parents HD2967 and HD2733 were crossed with the donor parent HD2932 to produce F_1 generations. F_1 plants (*Ne1ne1*) were backcrossed with respective recurrent parents to produce BC_1F_1 generations. The details of the marker-assisted backcrossing scheme followed in the current study are given in Fig. 1. Ten plants carrying the *ne1* allele and showing maximum phenotypic resemblance with their respective recurrent parents were selected in each backcross generation for marker-assisted background analysis. Plants with maximum recurrent parent genome (RPG) were backcrossed or selfed to produce the next generation. In BC_2F_2 generation, plants carrying *ne1* in

Table 1. Molecular markers used for Ne1and Ne2 alleles

Gene for necrosis	SSR markers	Primer sequence	Chromo some	Annealing temperature (^{0}C)	Dominant/Co-dominant	Reference
Ne1	*Xbarc74*	F:5′ GCGCTTGCCCCTTCAGGCGAG 3′ R:5′ CGCGGGAGAACCACCAGTGACAGAGC 3′	5BL	60	Co-dominant	Chu etal.(2006)
Ne2	*Leq54_LrLC10*	F: 5′CCACCAAACAAACTAAAGAAGC R: CACCCGATGACGATAAGC	2BS	58	Co-dominant	Zhang et al. (2022)
	Leq22_LrLC10	F: ACGTACAGAGAAGTGCCCAC R: GGCTCAAGTGGGTCTCTGAA	2BS	56	Co-dominant	Zhang et al. (2022)

a homozygous state and with maximum RPG recovery were identified. These plants were selfed to produce BC$_2$F$_3$ families. Percent genome recovery (PGR)in each backcross generation of HD2967 and HD2733 was calculated as given below:

Percent genome recovery (PGR) = [no of homozygous loci+1/2 (no of heterozygous loci)] / Total no of polymorphic primers (or) loci × 100

Marker analysis

Genomic DNA was isolated from 4 to 5-week-old seedlings by the CTAB method (Murray and Thompson, 1980). The DNA samples were quantified, and quality was confirmed with NanoDrop™ spectrophotometer. DNA samples were then diluted with millipore water to 25 ng/µL concentrations as working stock and stored at -20°C until used for PCR amplification. The PCR reaction was carried out in a reaction volume of 10 µL comprising 4 µL of 2x GoTaq PCR Master Mix (Promega, #M7122), 1-µL of each primer (5 pmol/µL), 2 µL of nuclease-free water and 2 µL of 25 ng/µL DNA (50 ng) in 96-well PCR plates with thermal seal in Eppendorf thermal cycler with following thermal profile: initial denaturation step of 94°C for 4 minutes, followed by 35 cycles of 94°C for 30 seconds (denaturation), at specific annealing temperature of primers for 30sec, 72°C for 30 seconds (primer extension) and a final extension of 72°C for 10 minutes and storage at 4°C. An agarose gel (3.5%) was prepared using high-gel-strength agarose to resolve the PCR products. Samples were loaded into wells using a micropipette. The gel was run at a voltage of 120 to 130 V for 2 to 3 hours. and visualized under UV light using a gel documentation system by Syngene.

Testcross with Ne2 carrier genotypes to know the efficiency of marker assisted selection

To determine the effectiveness of marker-assisted selection, plants homozygous for recessive (*ne1ne1*) and dominant (*Ne1Ne1*) alleles (in BC$_2$F$_2$ generation) were crossed with wheat cultivar Sonalika (*Ne2Ne2*), and test cross F$_1$s were produced. These test cross F$_1$s were raised to observe the development of necrosis in them.

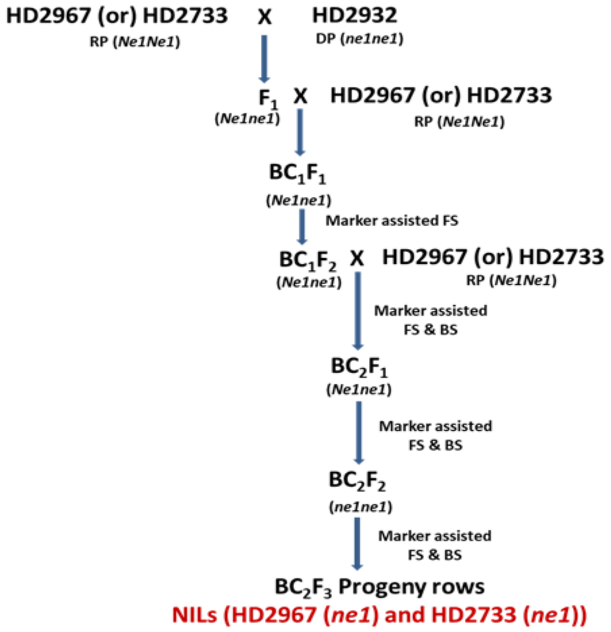

Fig. 1. Schematic diagram of development of non-necrotic near isogenic lines of HD2967 and HD2733 using marker-assisted backcross breeding

Results

Validation of markers for necrosis genes *Ne1* and *Ne2* and identification of necrosis genes present in wheat varieties HD2967 and HD2733 are done using the linked markers and the validated marker of *Ne1* was used in its marker-assisted elimation.

Validation of markers for the Ne1 gene

The markers reported to be linked with necrosis genes *Ne1* and *Ne2* (Table 1) were used for amplification in test genotypes using positive and negative checks for marker validation. Marker *Xbarc74*, linked with *Ne1*, produced three different bands of size 153, 165 and 179 bp. Wheat varieties, HD2967 and HD2733 and *Ne1* positive check C306 produced band of 165 bp (Fig 2) indicating presence of *Ne1* in HD2967 and HD2733. Wheat varieties, HD2932 (non-carrier for necrosis, i.e., *ne1ne1ne2ne2*) and Sonalika (carrier of *Ne2* i.e., *ne1ne1Ne2Ne2*), produced a band of size 153 bp compared to positive check C306 (*Ne1Ne1 ne2 ne2*)which produced 165 bp band. Negative checks, Agra Local and NI5439 also produced a band of 153 bp size (Fig. 2). Wheat genotypes HD3059, and Parula produced a band of 179 bp, indicating the absence of *Ne1* allele in both the genotypes.

Validation of marker for Ne2 gene

Indel markers*Leq54_LrLC10* and *Leq22_LrLC10* linked to necrosis gene *Ne2* were used in marker validation using the same set of genotypes as it was done in the case of the *Ne1* gene. Both the markers *Leq54_LrLC10* and *Leq22_LrLC10* produced similar banding patterns in Sonalika (positive check for *Ne2*) and test genotype Parula, indicating the presence of *Ne2* in Parula. All other genotypes (Agra Local, NI5439, C306, HD2967, HD2733, HD2932 and HD3059) produced different banding patterns compared to Sonalika and Parula indicated the absence of *Ne2* in all of them (Fig. 3). The presence/absence of necrosis genes *Ne1* and *Ne2* in each genotype is presented in Table 2.

Marker assisted elimination of Ne1 gene in wheat varieties HD2967 and HD2733

Screening of wheat genotypes HD2967 and HD2733 with marker *Xbarc74*, linked with *Ne1*, produced a band of 165 bp. Wheat variety C306, a known carrier of *Ne1*, also produced a similar band of 165 bp, indicating the presence of the *Ne1* allele in HD2967 and HD2733. Wheat variety HD2932 (*ne1ne1ne2ne2*), which was used as a donor parent, amplified a band of different sizes for both the markers of *Ne1* and *Ne2*, indicating the absence of both in HD2932.

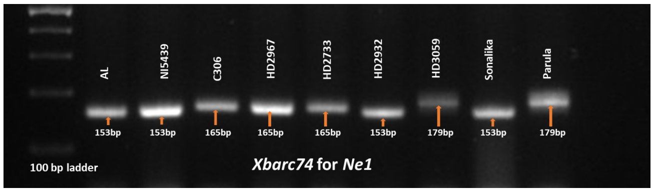

Fig. 2. Validation of molecular marker, *Xbarc74* linked to necrosis gene *Ne1* using a set of genotypes

Table 2. Presence/absence of necrosis genes *Ne1* and *Ne2* in test genotypes based on validated markers

S. No.	Genotype/Wheat variety	*Ne1 (+ve/-ve)*	*Ne2 (+ve/-ve)*
1.	Agra Local	-ve	-ve
2.	NI5439	-ve	-ve
3.	C306	+ve	-ve
4.	HD2967	+ve	-ve
5.	HD2733	+ve	-ve
6.	HD2932	-ve	-ve
7.	HD3059	-ve	-ve
8.	Sonalika	-ve	+ve
9.	Parula	-ve	+ve

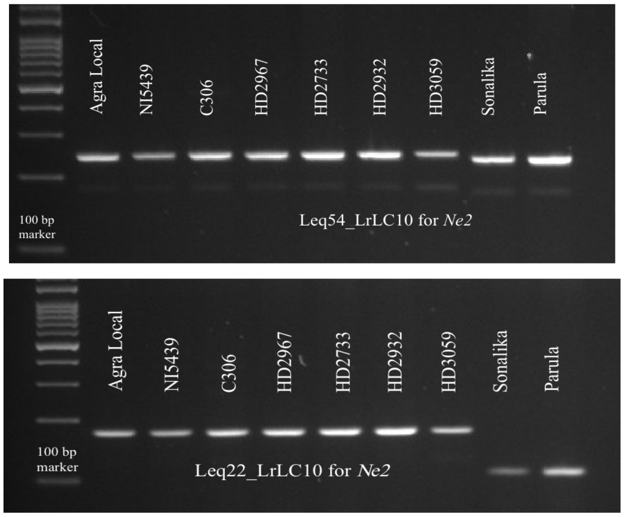

Fig. 3. Validation of molecular marker, *Leq54_LrLC10* and *Leq22_LrLC10* linked to necrosis gene *Ne2* using a set of genotypes

A parental polymorphism survey between the donor genotype for the *ne1* allele, HD2932, and recurrent parents HD2967 and HD2733 identified 48 and 45polymorphic markers, respectively, out of 642 markers used. The percentage of polymorphic markers was 7.47 and 7.00% in HD2967 and HD2733, respectively. These polymorphic markers were used in the background selection of plants carrying recessive allele *ne1* in each backcross generation.

BC$_1$F$_1$ generation derived from the cross HD2967/HD2932 was screened with linked marker *Xbarc74*. Of, 200 BC$_1$F$_1$ plants, 92 plants showed the presence of *ne1* in a heterozygous state (*Ne1ne1*) (Table 3). These plants were subjected to phenotypic selection. Ten plants with maximum phenotypic similarity with recurrent parents were selfed to produce BC$_1$F$_2$ generation. Foreground selection of 43 BC$_1$F$_2$ plants identified 11 plants with *ne1* in the homozygous state (*ne1ne1*) and 17 plants with *ne1* in the heterozygous state (*Ne1ne1*) (Table 3). Phenotypic and background selection in BC$_1$F$_2$ generation identified single heterozygous plants (*Ne1ne1*) with a maximum of 85.10% of RPG. This plant was backcrossed with recurrent parent HD2967 to produce BC$_2$F$_1$ generation. Foreground selection of 90 BC$_2$F$_1$ plants identified 51 plants with the *ne1* allele in a heterozygous state (Table 3). Background selection of 10 phenotypically selected BC$_2$F$_1$ plants (with *Ne1ne1*) identified a plant with 95.75% RPG (Table 3). The plant with maximum RPG was selfed to produce BC$_2$F$_2$ generation. A total of 40 BC$_2$F$_2$ plants were produced. In the BC$_2$F$_2$

generation foreground selection with co-dominant marker *Xbarc74* identified 9 plants as homozygous and 23 plants as heterozygous for *ne1* allele. Background selection in homozygous plants identified plants with a maximum RPG of 96.80%. BC_2F_2 plants homozygous for *ne1* were selfed to produce BC_2F_3 families.

Like HD2967, backcross generations of HD2733 were selected using foreground and background markers for identifying homozygous NILs with maximum RPG of HD2733. Foreground selection of 150 BC_1F_1 plants from the cross HD2733/HD2932 identified 73 plants with *ne1* in a heterozygous state (*Ne1ne1*) (Table 3). These plants were subjected to phenotypic selection. Ten plants with maximum phenotypic similarity with recurrent parent were selfed to produce BC_1F_2 generation. Foreground selection in BC_1F_2 generation identified 21 homozygous (*ne1ne1*) and 22 heterozygous (*Ne1ne1*) plants out of 68 plants screened (Table 3). Phenotypic and background selection in BC_1F_2 generation identified single heterozygous plants (*Ne1ne1*) with a maximum of 84.78% of RPG and backcrossed to HD2733 to produce BC_2F_1 generation. Foreground selection of 302 BC_2F_1 plants identified 124 plants with *ne1* in heterozygous state (*Ne1ne1*) (Table 3). Background selection of 10 phenotypically selected BC_2F_1 plants identified a plant with 93.47% of RPG (Table 3). This plant was selfed to produce BC_2F_2 generation. Foreground selection of 90 BC_2F_2 plants identified 15 plants homozygous and 53 plants heterozygous for *ne1* allele. A representative gel picture of foreground selection for *ne*$_1$ with marker *Xbarc74* in BC_2F_2 generation is presented in Fig. 4. Background selection in homozygous plants identified plants with maximum RPG of 95.55%. BC_2F_2 plants homozygous for *ne1* were selfed to produce BC_2F_3 families.

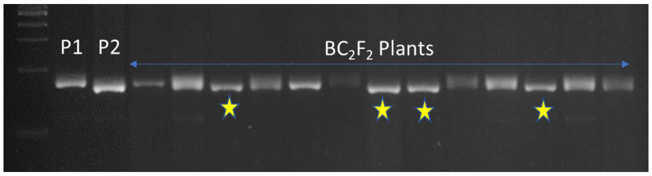

Fig. 4. Foreground selection in BC_2F_2 generation of cross HD2733/HD2932 using marker *Xbarc74*. Here P1, HD2733; P2, HD2932 and

Result of test crosses with Ne2 carriers

The testcross F_1s generated from BC_2F_2 plants identified as homozygous for *ne1* and *Ne1* were raised and the seedlings were observed for development of necrosis. The F_1s produced from the crosses, HD2733 (*ne1*) × Sonalika (*Ne2*) and HD2967 (*ne1*) × Sonalika (*Ne2*) showed normal seedlings without any necrosis while the F_1s produced from the crosses HD2733 (*Ne1*) × Sonalika (*Ne2*) and HD2967 (*Ne1*) × Sonalika (*Ne2*) produced complete necrosis in all the seedlings.

Discussion

Bread wheat varieties HD2967 and HD2733 have been the dominant varieties cultivated in different wheat-growing zones of India. During the hybridization program under different wheat improvement projects, it was observed that these varieties were producing hybrid necrosis in several crosses. When crossed with Parula (a donor of APR genes *Lr34*, *Lr46*, and *Lr68*), these wheat varieties produced hybrid necrosis. Since many of the donor genotypes involved in crossing carried the *Ne2* gene for necrosis, it became difficult to utilize these high-yielding varieties in the crossing programme. Therefore, the present study was initiated to substitute the dominant allele *Ne1* in HD2967 and HD2733 with the recessive allele *ne1* so that they can be crossable with any genotype without producing hybrid necrosis. Though markers for both *Ne1* and *Ne2* are available, only one report was available where a marker of *Ne2* was used in the screening of wheat cultivars. Apart from this, there are no such reports available where markers of necrosis genes were used either for screening or for marker-assisted selection.

Table 3. No. of plants identified of carrying *ne1* allele in each backcross generation using markers *Xbarc74* and their percent recurrent parent genome recovery

Breeding cross	Generation	No. of plants screened	Number of plants carrying ne1 allele		Maximum recurrent parent genome (RPG)%
			In homozygous condition	In heterozygous condition	
HD2967/ HD2932	BC_1F_1	200	-	92	-
	BC_1F_2	43	11	17	85.10
	BC_2F_1	90	-	51	95.75
	BC_2F_2	40	9	23	96.80
HD2733/ HD2932	BC_1F_1	150	-	73	-
	BC_1F_2	68	21	22	84.78
	BC_2F_1	302	-	124	93.47
	BC_2F_2	90	15	53	95.55

The necrosis genes, *Ne1* and *Ne2*, are well characterized, and markers linked to both genes are available (Chu et al. 2006; Li et al. 2021). The co-dominant marker *Xbarc74*, linked with *Ne1,* was validated on a set of bread wheat genotypes. It produced a band of size 165 bp in wheat varieties HD2967, HD2733, and C306 (positive check for the dominant allele *Ne1*), while wheat genotypes Agra Local, NI5439, HD2932, and Sonalika produced a band of 153 bp and HD3059 and Parula produced 179 bp bands, indicating presence of *Ne1* in HD2967 and HD2733 only and its absence in other genotypes. Also, the 165 bp band amplified by *Xbarc74* is corresponding to the *Ne1* allele. Amplification of bands of different sizes with one marker may correspond to the presence of multiple alleles at a single locus. Galaiev (2016) studied the distribution of different alleles of necrosis gene *Ne2* in different wheat cultivars of Ukrainian and Russian selections using marker *Xbarc55*-2B. It produced 142 bp for *ne2*, 136 bp for $Ne2^{w/m}$, 132 bp for $Ne2^{ms}$ and 126 bp for $Ne2^{s}$.

Indel markers *Leq54_LrLC10* and *Leq22_LrLC10* linked to necrosis gene *Ne2* were used in the present study for initial validation. Both the markers produced unique bands in positive checks Sonalika and Parula, which carried *Ne2* The other genotypes AL, NI5439, C306, HD2967, HD2733, HD2932 and HD3059 produced bands of different sizes. Wheat genotypes C306, HD2967, and HD2733 carry dominant *Ne1* while AL, NI5439, HD2932, and HD3059 are non-carriers for necrosis and carry both the genes in recessive homozygous condition. i.e., *ne1ne1ne2ne2*. Thus, the SSR marker *Xbarc74* and the two indel markers *Leq54_LrLC10* and *Leq22_LrLC10* were validated to identify alleles for *Ne1* and *Ne2* genes. Vikas et al. (2013) also identified the presence of *Ne1Ne1ne1ne1*gene in HD2733, *ne1ne1Ne2Ne2* gene in Sonalika and both genes in recessive condition (*ne1ne1ne2ne2*) in NI5439 by crossing these lines with C306 and HD2329, known carriers of necrosis genes *Ne1Ne1ne2ne2* and *ne1ne1Ne2Ne2*, respectively.

The validated markers were used in the marker-assisted breeding programme in the present study to develop Near Isogenic Lines (NILs) of HD2967 and HD2733, which carried the recessive allele *ne1* instead of the dominant allele *Ne1*. Different backcross generations (BC_1F_1, BC_1F_2, BC_2F_1, BC_2F_2, and BC_2F_3) were raised and foreground and background selections were conducted as per the standard procedure. The wheat variety HD2932 was used as a donor variety for the *ne1* allele. HD2932 has been extensively used in our breeding programme without producing necrosis (Mallick et al. 2015, 2022, and 2024) and found to carry two necrosis genes in homozygous recessive condition i.e.,*ne1ne1ne2ne2*. The markers used for necrosis genes *Ne1* and *Ne2* in the present study also confirmed this. Marker-assisted background selections coupled with phenotypic selection in every backcross generation helped to recover 96.80% of RPG in HD2967 and 95.55% of RPG in HD2733 in their BC_2F_2 generations. Marker-assisted background selection in phenotypically selected plants in each backcross generation has always helped to recover more than 90% of RPG in just two backcrosses and one generation of selfing (Mallick et al. 2015, 2022a, b and 2024). Also, as both the donor and recurrent parents used here are cultivated wheat varieties, recovery of RPG was quite easy due to identification of lesser number of polymorphic markers between them. To validate the results of marker-assisted elimination of *Ne1* gene in HD2967 and HD2733, the BC_2F_2 plants with homozygous recessive (*ne1*) and dominant (*Ne1*) alleles were crossed with *Ne2* carrier Sonalika. As expected, F_1s from HD2967 (*Ne1*)/ Sonalika (*Ne2*) and HD2733 (*Ne1*)/Sonalika (*Ne2*) cross produced necrosis and all the seedlings died. On the other hand, F_1s of Sonalika (*Ne2*) with NILs of HD2967 and HD2733 carrying recessive allele *ne1*, grew normally without producing necrosis. Thus the test crosses of newly developed lines of HD2967 and HD2733 carrying *ne1* allele validated the results of marker-assisted selection. NILs of HD2967 and HD2733 carrying the *ne1* allele in homozygous state are useful genetic resources that can be freely used in crossing programmes as these lines are now non-carriers for necrosis genes.

Authors' contribution

Conceptualization of research (NM, V); Designing of the experiments (SKJ, NM, MSS); Contribution of experimental materials (V, NM); Execution of field/lab experiments and data collection (BSV, SB, MKC); Analysis of data and interpretation (AKC, PA, RK); Preparation of the manuscript (NM, V, SKJ, NMS).

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