

RESEARCH ARTICLE



Marker-assisted pyramiding of *Thinopyrum*-derived leaf rust resistance genes *Lr19* and *Lr24* in bread wheat variety HD2733

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Abstract. This study was undertaken to pyramid two effective leaf rust resistance genes (*Lr19* and *Lr24*) derived from *Thinopyrum* (syn. *Agropyron*), in the susceptible, but agronomically superior wheat cultivar HD2733 using marker-assisted selection. In the year 2001, HD2733 was released for irrigated timely sown conditions of the north eastern plains zone (NEPZ) of India became susceptible to leaf rust, a major disease of the region. Background selection helped in developing near-isogenic lines (NILs) of HD2733 with *Lr19* and *Lr24* with 97.27 and 98.94%, respectively, of genomic similarity with the parent cultivar, after two backcrossing and one generation of selfing. NILs were intercrossed to combine the genes *Lr19* and *Lr24*. The combination of these two genes in the cultivar HD2733 is expected to provide durable leaf rust resistance in farmers' fields.

Keywords. wheat; leaf rust; molecular marker; gene pyramiding; marker-assisted selection.

Introduction

Rust diseases caused by *Puccinia* spp. inflict significant yield losses to wheat crop throughout the world (Kolmer 2005; Tomar *et al.* 2014). There are three rusts, leaf rust (*Puccinia triticina*), stem rust (*Puccinia graministritici*) and stripe rust (*Puccinia striiformis*). All three prevail in India, but require different agroecological conditions for disease development. Leaf rust exists in all the wheat growing regions, while stem rust is common in warmer areas of central and peninsular India. Stripe rust is a disease of cooler areas of north western India including northern Himalayas. Development and deployment of rust-resistant cultivars is the most economical, effective and environment friendly approach to prevent the damage caused by rust diseases (Kolmer 1996). So far, 76 leaf rust resistance (*Lr*) genes have been designated (McIntosh *et al.* 2016). About half of them are native to the bread wheat *Triticum aestivum*, but most are not effective to existing Indian

racess. Therefore, alien genes for leaf rust resistance have been deployed in many cultivars, which provide effective resistance. However, some of the alien leaf rust resistance genes such as *Lr9*, *Lr19*, *Lr26* and *Lr28* became ineffective due to evolution of new virulent races (Nayar *et al.* 2003; Bhardwaj *et al.* 2005, 2010). Of these, only *Lr26* is used extensively in wheat cultivars. In general, cultivars carrying single race specific resistance gene were found to be short-lived due to evolution of new races of pathogen. Therefore, pyramiding of rust resistance genes is considered an effective strategy for enhancing durability of resistance genes.

Wheat variety HD2733 is a high-yielding variety released for cultivation under timely sown irrigated conditions of NEPZ in India. However, over a period of time, this cultivar became susceptible to leaf rust. To enhance durability of HD2733, efforts were initiated to transfer rust resistance genes using marker-assisted backcross breeding (MABB). While conventional backcross method requires 6–7 generations to transfer targeted genes in a variety, MABB

can achieve similar results in 2–3 generations of backcrossing (Pederson and Leath 1988; Mallick et al. 2015). Moreover, identifying the plants carrying the targeted resistance gene(s) in each backcross generation is easier using molecular markers than the conventional method of selection. In this paper, we report the accelerated transfer of *Lr19* and *Lr24* rust resistance genes using marker-assisted foreground and background selections in wheat variety HD2733. Since *Lr19* and *Lr24* are linked with stem rust resistance genes *Sr25* and *Sr24*, respectively, the pyramided lines are expected to carry them as well.

Materials and methods

Plant materials

Wheat variety HD2733 was used as recipient parent. Donor genotypes included two backcross derived lines of bread wheat variety HD2687 carrying resistance genes for leaf rust, *Lr19* (HD2687+*Lr19*) and *Lr24* (HD2687+*Lr24*), respectively (Sivasamy et al. 2007).

Molecular markers and marker analysis

SSR marker *Xwmc221* and SCAR marker *SCS1302* were used for marker-assisted foreground selection of *Lr19* and *Lr24*, respectively (Gupta et al. 2006a, b). For background selection, 907 SSR markers spanning across 21 chromosomes of bread wheat were used for parental polymorphism. Recurrent parent genome (RPG) was calculated as described earlier (Mallick et al. 2015).

The polymerase chain reaction (PCR) was carried out in 10 μ L reaction volumes with 25 ng of genomic DNA, 1.0 unit *Taq* DNA polymerase (Bangalore Genei, Bengaluru, India), 200 μ M of each dNTP (MBI Fermentas, Germany), 0.2 μ M of both forward and reverse primers, 4 mM Tris-HCl (pH 8.0), 20 mM KCl and 0.8 mM MgCl₂. The PCR was performed in thermal cycler (Eppendorf, model Mastercycler pro S, Hamburg, Germany) at temperature profile of 94°C for 4 min, followed by 35 cycles of reaction having 94°C for 30 s, 50–60°C for 30 s (annealing temperature depending on primer) and 72°C for 30 s, with a final extension at 72°C for 10 min. The amplified products were resolved on MetaPhor (Lonza) agarose gel (3.5%) and visualized by ethidium bromide staining.

DNA extraction

Leaf tissues for genomic DNA isolation were collected after 30–40 days of sowing. DNA was isolated by CTAB method (Murray and Thompson 1980) and quantified on 0.8% agarose gel by comparing samples with 100/200 ng of Lambda uncut DNA. Working DNA stocks were prepared by diluting in TE buffer to achieve final concentration of about 25 ng/ μ L for PCR amplification.

Marker-assisted gene pyramiding scheme

HD2733 was crossed with donor lines HD2687+*Lr19* and HD2687+*Lr24* to produce two separate F₁ generations in 2009–2010. F₁ plants were crossed with recurrent parent HD2733 to develop BC₁F₁ generation. Marker-assisted selection for *Lr19* and *Lr24* was carried out in respective BC₁F₁ generations. Gene positive plants in BC₁F₁ were subjected to background analysis. Plants with targeted leaf rust resistance gene with maximum RPG were backcrossed with recurrent parent HD2733 to generate two separate BC₂F₁ populations. In BC₂F₁, foreground as well as background selections were practiced as done in BC₁F₁. BC₂F₁ plants with rust resistance genes and maximum RPG were selfed as well as intercrossed to obtain BC₂F₂ and NILF₁ generations, respectively (figure 1). Marker-assisted foreground selection identified the plants homozygous for *Lr19* and those carrying *Lr24* in homozygous/heterozygous state, in respective BC₂F₂ populations. Selected BC₂F₂ plants with *Lr24* were selfed to develop BC₂F₃ families. Nonsegregating BC₂F₃ families, carrying *Lr24* in homozygous state were identified by *Lr24*-linked marker. Plants carrying single leaf rust resistance gene in homozygous state in BC₂F₂ generation were subjected to background analysis and plants with maximum RPG (NILs) were identified. The intercrossed NILF₁ plants carrying both *Lr19* and *Lr24* were selfed to produce NILF₂ generation. NILF₂ population was raised and plants carrying *Lr19* in homozygous state along with *Lr24* were identified with respective markers. NILF₂ plants homozygous for *Lr24* were identified on the basis of progeny testing in NILF₃. Thus, NILF₃ families carrying both *Lr19* and *Lr24* in homozygous state were developed. The details of gene pyramiding scheme are given in figure 1.

Apart from the use of molecular markers, shuttle breeding was used to accelerate the development of NILs. Two generations in a year were raised; one at IARI, New Delhi, India, to grow main season crop in winter and the other at IARI, Regional Station, Wellington, Tamil Nadu, India, to grow off-season crop in summer. The main season at Delhi was used to raise F₁, BC₂F₁, BC₂F₃, NILF₂ and NILF₃ generations, while BC₁F₁, BC₂F₂ and intercrossed NILF₁ were grown in off-season at Wellington (Nilgiri hills) (figure 2).

Rust inoculation

Segregating material was tested with the most dominant leaf rust race 77-5 under artificial epiphytotic conditions. NILF₃s were tested in two isolated nurseries inoculated with mixture of leaf and stem rust races. The generations raised at Wellington were naturally exposed to leaf and stem rusts, as Wellington is a natural hot spot for the two rusts (Nagarajan et al. 1980). The rust reactions were recorded at adult plant stage following modified Cobb

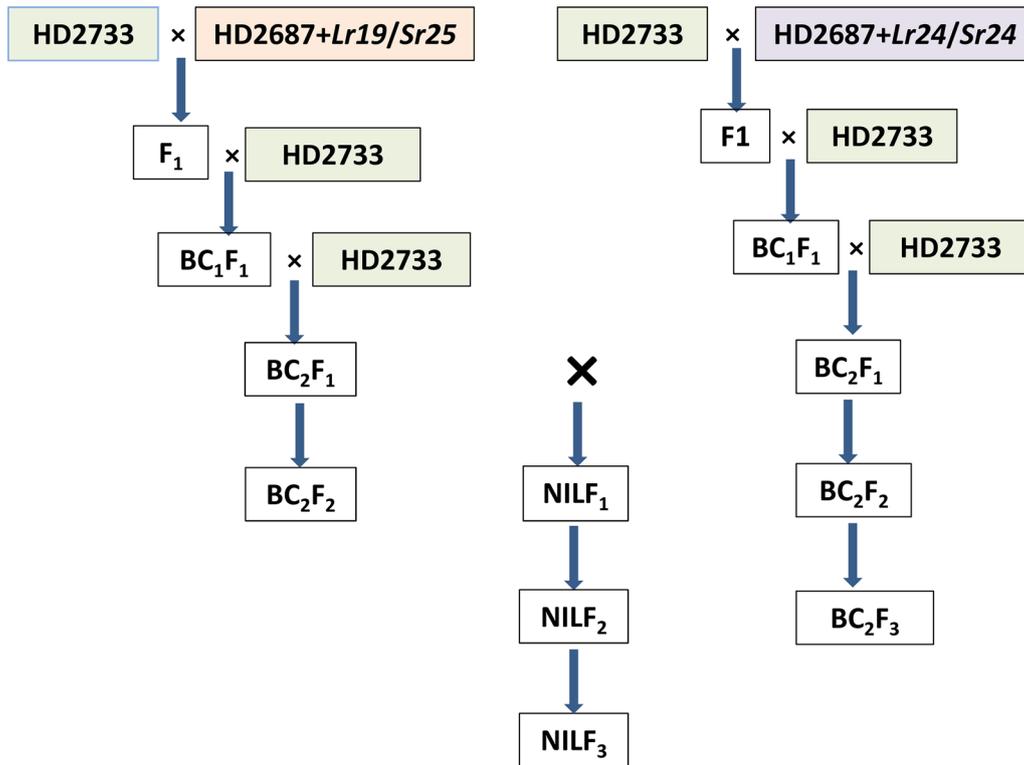


Figure 1. Marker-assisted gene pyramiding scheme to combine leaf rust-resistance genes *Lr19* and *Lr24* in wheat variety HD2733.

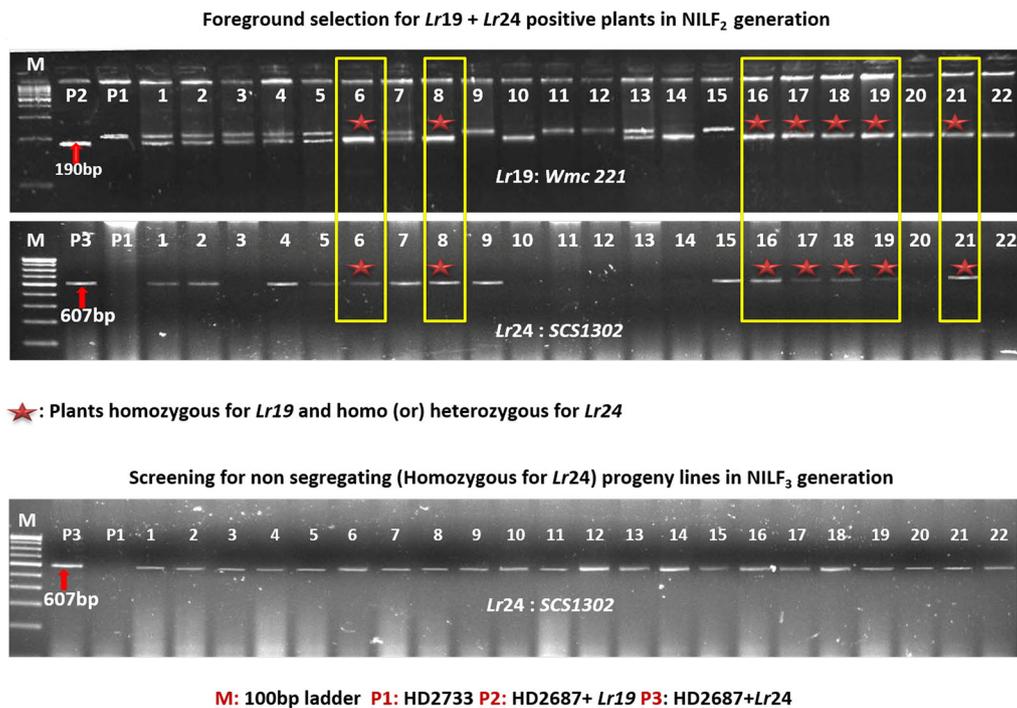


Figure 2. Foreground selection for *Lr19*+*Lr24* positive plants homozygous for both the genes.

Scale (Peterson *et al.* 1948), where rust severity is recorded on 0–100 scale along with infection response as S, susceptible; MS, moderately susceptible; MR, moderately resistant; TR, trace (Joshi *et al.* 1988).

Results

Genetic analysis of recurrent (HD2733) and donor parents (HD2687+*Lr19* and HD2687+*Lr24*) using 907 SSR

markers, identified 110 and 95 markers, respectively, as polymorphic. Polymorphic markers were used for background selection of plants carrying targeted resistance genes. The number of plants subjected to background selection was minimized based on phenotypic similarity with the recurrent parent HD2733.

In BC₁F₁ (HD2733/HD2687+*Lr19*//HD2733), 62 plants were identified carrying *Lr19* in heterozygous state by marker *Xwmc221*. In the other BC₁F₁ (HD2733/HD2687+*Lr24*//HD2733), SCAR marker *SCS1302* identified 64 plants with *Lr24* (table 1). All the selected plants were resistant in the field with no visible symptoms of leaf rust, while the recurrent parent HD2733 showed susceptible reaction of 40–50 S. Background selection with 110 polymorphic markers in *Lr19* positive plants in BC₁F₁ recovered a maximum of 81.03% RPG. Similarly, background analysis with 95 polymorphic markers in the other BC₁F₁ population (HD2733/HD2687+*Lr24*//HD2733) identified maximum genomic similarity of 87.67% with HD2733 (table 1). Two plants from each BC₁F₁ population with targeted gene and high RPG were backcrossed with HD2733 to minimize the risk of losing any relevant plant. In BC₂F₁, 30 plants carrying *Lr19* and 15 plants carrying *Lr24*, were identified (table 1). As expected, all the plants carrying *Lr19* were heterozygous in BC₁F₁ and BC₂F₁. However, for *Lr24*, the presence of the gene could be established in BC₁F₁ and BC₂F₁ generations, since unlike *Lr19* marker *Xwmc221*, the SCAR marker for *Lr24* is a dominant marker.

In the BC₂F₁, recovery of HD2733 genome increased to 91.95 and 91.09% for the *Lr19* and *Lr24* gene positive plants, respectively. The BC₂F₂ foreground analysis revealed that 16 plants of 51 were homozygous for *Lr19* and 31 plants of 55 carried *Lr24*, either in homozygous or heterozygous state. Background analysis showed maximum RPG of 97.27% for *Lr19* and 98.94% for *Lr24* in BC₂F₂. In this way, two NILs of HD2733 individually carrying *Lr19* and *Lr24* were developed. Screening of 40 NILF₁ plants produced by intercrossing the two BC₂F₁s for *Lr19* and *Lr24* with respective markers identified seven plants carrying both the genes. These seven plants were selfed to produce NILF₂ generation. Foreground analysis of selected 130 NILF₂ plants identified 53 plants carrying *Lr19* in homozygous condition along with *Lr24*. The two gene combination plants in NILF₂ were subjected to background analysis which revealed a maximum RPG recovery of 98.57%. Of the 53 NILF₂ plants, 12 were found homozygous for both *Lr19* and *Lr24* based on progeny testing in NILF₃. The NILs carrying *Lr19*, *Lr24* and *Lr19*+*Lr24* were evaluated for leaf rust resistance and stem rust resistance. All the NILs showed high degree of resistance to leaf rust with maximum disease reaction of TR to 10 MR. Additionally, NILs also showed resistance to stem rust, reaction ranging from 5 S to 5 MR. The recurrent parent HD2733 showed susceptible reaction (40 S) to leaf rust and moderately susceptible reaction (10 MS) to stem rust (table 2).

Table 1. Number of gene positive plants and maximum RPG% identified in different generations of HD2733.

Cross	Generation	Total no. of plants screened	No. of gene positive plants		No. of gene negative plants	Maximum RPG (%)
			Homozygous	Heterozygous		
1 HD2733/ HD2687 + <i>Lr19</i>	BC ₁ F ₁	128	–	62	66	81.03
	BC ₂ F ₁	75	–	30	45	91.95
	BC ₂ F ₂	51	16	21	14	97.27
2 HD2733/ HD2687 + <i>Lr24</i>	BC ₁ F ₁	134	–	64	70	87.67
	BC ₂ F ₁	32	–	15	17	91.09
	BC ₂ F ₂	55		31	24	98.94
	BC ₂ F ₃	31	12	19	–	–
			Number of plants with two gene combinations			
	Generation	Total no. of plants screened	Homozygous	Heterozygous	No. of gene negative plants	Maximum RPG (%)
3 HD2733+ <i>Lr19</i> /HD2733+ <i>Lr24</i>	NILF ₁	40	–	7	33	–
	NILF ₂	130		53	–	98.57
	NILF ₃	53	12	41	–	–

Table 2. Maximum rust reaction recorded in the recurrent parent HD2733 and selected NILs.

Introgressed lines	Maximum rust infection	
	Leaf rust	Stem rust
HD2733	40 S	10 MS
HD2733+ <i>Lr19/Sr25</i>	10 MR	15 MS
HD2733+ <i>Lr24/Sr24</i>	10 MR	5 S
HD2733+ <i>Lr19/Sr25 + Lr24/Sr24</i>	TR	5 MR

Discussion

HD2733 is a well-adapted and high yielding wheat variety in NEPZ of India. Since, it became susceptible to leaf rust, its resistance was restored by pyramiding two resistance genes *Lr19* and *Lr24*. The *Thinopyrum*-derived resistance gene *Lr19* (Sharma and Knott 1966) is linked with stem rust resistance gene *Sr25*. Singh *et al.* (1998) reported that 7D/7Ag translocation carrying *Lr19/Sr25* enhanced grain yield by 10–15% in several genotypes. The other *Thinopyrum*-derived leaf rust resistance gene *Lr24* present on the long arm of 3D chromosome is linked with stem rust resistance gene *Sr24* (McIntosh *et al.* 1976). Thus, besides pyramiding of leaf rust resistance genes *Lr19* and *Lr24*, stem rust resistance genes *Sr25* and *Sr24* were also pyramided simultaneously. Additionally, stem rust resistance gene *Sr25* confers resistance to stem rust race Ug99 (Sharma *et al.* 2013). Though, race Ug99 is not present in India, it is desirable to incorporate genetic resistance against Ug99 to ward off future threats. The combination of *Lr19/Sr25+Lr24/Sr24* confers resistance to all the prevalent races of leaf and stem rusts in India. The first wheat variety carrying *Lr24* was released in 1993 and subsequently a large number of varieties with *Lr24* have been released in India. Although, virulence has been reported from several countries, *Lr24* continues to provide effective resistance in India (Tomar *et al.* 2014). For *Lr19*, a race (77-8) was reported as virulent. However, it is a weak race and despite of it being reported in 2005, it has not spread in India. HD2733 pyramided lines carrying both *Lr19* and *Lr24* are expected to cross protect each other, thus enhancing their durability. *Lr24* does not seem to impose yield penalty as demonstrated by the fact that several cultivars with *Lr24* have been released for cultivation in India (Tomar *et al.* 2014).

Transfer of rust resistance genes by conventional backcross method requires screening with discriminating races either at seedling or adult stage under epiphytotic conditions. However, with the availability of robust molecular markers linked with resistance genes, it is possible to identify targeted resistance gene(s) in segregating populations with greater precision (Sivasamy *et al.* 2009; Revathi *et al.* 2010; Bhawar *et al.* 2011). At the same time, background selection with molecular markers enables faster recovery of genome of the recipient variety.

In the present experiment, background selection using polymorphic markers was started right from BC₁F₁ generation. Phenotypic selection of plants similar to recurrent parent HD2733 followed by marker-assisted background selection helped in faster recovery of RPG. Parental polymorphism between HD2733 and the two donor lines, HD2687+*Lr19* and HD2687+*Lr24* identified markers which helped in recovering alleles specific to HD2733 in each backcross generation. The number of markers required for background selection in BC₂F₁ and BC₂F₂ reduced considerably, hence, after each backcrossing, some alleles of HD2733 get fixed, so that no further selection is required. Using MABB, it was possible to select desirable plants with high RPG along with resistance genes in each generation, which in turn accelerated the development of NILs of HD2733.

Marker-assisted selection

Foreground selection was carried out in BC₁F₁, BC₂F₁, BC₂F₂, NILF₁ and NILF₂ generations to identify plants with *Lr19* and *Lr24*. Plants homozygous for *Lr19* were identified in BC₂F₂ using codominant marker *Xwmc221*. However, SCAR marker *SCS1302* being dominant, it was not possible to identify plants homozygous for *Lr24*. Plants homozygous for *Lr24* were identified on the basis of progeny testing in BC₂F₃ generation. Near-isogenic lines carrying *Lr19* and *Lr24* individually were intercrossed to combine targeted genes *Lr19* and *Lr24* in NILF₁. Marker-assisted selection with *Lr19* and *Lr24* was also conducted in intercrossed (NILF₁) plants as the combination cross was made in BC₂F₁ itself, where both the genes were in heterozygous state. The plants identified in NILF₁ generation were heterozygous for both *Lr19* and *Lr24*. In NILF₂, plants homozygous for *Lr19* and additionally carrying *Lr24* were identified using respective molecular markers. In NILF₃, families homozygous for *Lr24* were identified. Since these families were already homozygous for *Lr19*, it was possible to select the F₃ families carrying both *Lr19* and *Lr24* in homozygous state.

Marker-assisted background selection accelerated the recovery of RPG. In BC₁F₁ derived from the cross HD2733/HD2687+*Lr19*//HD2733, maximum 81.03% genomic similarity with recurrent parent HD2733 was recovered. In the BC₂F₁, the genomic similarity increased to 91.95%, which further increased to 97.27% in BC₂F₂. Thus, NILs carrying *Lr19* were produced with two backcrosses followed by one generation of selfing. The other leaf rust resistance gene *Lr24* was also transferred efficiently in the background of HD2733. Plants with highest RPG were identified in the BC₁F₁, BC₂F₁ and BC₂F₂ generations (table 1). Thus, NILs of HD2733 carrying genes *Lr19* and *Lr24* with RPG of NILs reaching to 97.27 and 98.94%, respectively, were developed. Phenotypic selection was used in addition to marker-assisted

background selection, which helped in recovering better plant types.

The NILs carrying individual genes *Lr19* and *Lr24* can be used in future for producing other gene combinations. Higher recovery of the HD2733 genome (more than 90% RPG) in the BC₂F₁ generation also enabled intercrossing *Lr19* and *Lr24* carrying plants to produce two gene combinations. Combination of marker-assisted foreground and background selections significantly reduced the period required to produce NILs carrying *Lr19* and *Lr24* individually. Two different approaches are recommended to pyramid two or more rust resistance genes in a popular cultivar. In the first approach, genes from different donors were first combined in a single plant, followed by backcrossing with recurrent parent and selection of targeted genes. In the second approach, which was followed in the present study, NILs carrying individual genes were produced first, followed by intercrossing of NILs to combine the targeted genes. Ishii et al. (2008) showed that the second approach, where NILs are produced is superior to the first approach. The recurrent parent HD2733 carries IRS. IBL translocation which carries *Lr26* and *Sr31*. *Lr26* is not effective in India, therefore, HD2733 showed susceptible reaction to leaf rust. However, moderately susceptible response (10 MS) of HD2733 against stem rust (table 2) is due to the presence of *Sr31*. The NILs developed in the present study provide improved versions of HD2733 with high degree of leaf rust resistance and stem rust resistance.

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