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Combining multiple rust resistance genes by phenotypic and marker assisted selection in wheat (*Triticum aestivum* L.)

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Abstract

Development of cultivars with genetic resistance has been the most effective and economical strategy to control rust diseases of wheat (*Triticum aestivum* L.). Leaf rust (*Puccinia triticina*) and stripe rust (*Puccinia striiformis*) are the two most important diseases of wheat in India. Combining two or more major rust resistance genes into one highly adapted cultivar is one of the important strategies for obtaining durable resistance. Molecular markers have made it possible to identify and pyramid valuable genes of agronomic importance in resistance breeding. The leaf rust resistance genes *Lr24*, *Lr28* and stripe rust resistance gene *Yr15* were pyramided into a popular but rust susceptible wheat variety HD2687 using marker assisted backcross breeding. SCAR markers $SCS1302_{607}$ for *Lr24* and $SCS421_{570}$ for *Lr28* and SSR marker Xgwm273 for *Yr15* gene were used to select gene positive plants. Different combinations of the three resistance genes were selected in BC_4F_2 generation of HD2687 with the help of linked molecular markers. Agronomic performance of improved lines was compared with that of the recipient parent.

Key words: Wheat, leaf rust, stripe rust, molecular markers, gene pyramiding

Introduction

Among the diseases of wheat, rusts caused by fungal pathogens are prominent. The three important rusts of wheat, black or stem rust caused by *Puccinia graminis* Pers.f.sp *tritici* Eriks. & Henn., brown or leaf rust caused by *Puccinia triticina* Eriks. (Syn: *Puccinia recondita*) and yellow or stripe rust incited by *Puccinia striiformis* Westend are known to cause significant yield losses [1] when they appear in epidemic proportion. Among these, stripe rust is the most common in both winter and spring wheat growing in cooler and humid regions of production such as in West Asia, Eastern Africa, China, South America and North-western Europe. Leaf rust causes serious damage to wheat varieties grown in comparatively warm and humid environment such as, South Asia, North Africa, Southeast Asia and South America, whereas black rust has been a potential threat to wheat production in North America, Australasia, Northern Africa, South Africa and some areas of Europe where temperatures are slightly higher [2]. Controlling rusts is a difficult task because of their fast multiplication and mutation abilities to change the strains (races) which overcome the effective genes in short span of time. The methods employed for disease control are the use of fungicides and cultivation of resistant varieties. However, chemical control of rust pathogens is inefficient, expensive and also not ecofriendly. Growing resistant varieties is the most economic, effective and environment-friendly approach to control the rust disease [3, 4].

Wheat production largely depends on the resistance imparted by the diverse and well characterized genes. Qualitative resistance, which confers major gene-specific resistance against some pathogen races, is easiest to incorporate into genotypes and is usually considered a gene-for-gene type of resistance. Several rust resistance genes have been transferred in the genetic background of popular Indian cultivars using conventional backcross breeding approaches [5]. To date, nearly 57 stem rust resistances, 71 leaf rust resistances and about 53 stripe rust resistance genes have been identified in

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wheat and related species [6]. The use of cultivars with single gene resistance permits the rapid evolution of virulence in the pathogen rendering the resistance ineffective in relatively short time. Therefore it is important to build a strategy that provides a greater durability of resistance in a variety. Gene pyramiding, a breeding procedure of bringing together more than one resistance gene into one desirable genetic background is theoretically attractive and one of the most economic approaches for long field survival of a variety without getting affected by the disease. When two resistance genes conferring resistance against the pathogen are combined in a genotype, the occurrence of two simultaneous mutations overcoming the resistance of such a cultivar would be unlikely. The selection of genotypes carrying two or more genes using traditional host-parasite interaction is very time consuming and often not possible due to lack of isolates with specific virulence and difficulty of identifying one resistance gene in the presence of another gene. Since the identity of the two resistance genes is difficult through conventional genetic analysis, application of molecular marker technology can tackle such complex problems. Marker assisted pyramiding approach has been earlier used in wheat and other crops [7-11]. HD2687 is a highly adapted cultivar with high yield potential. It carries 1BL/1RS translocation of *Secale cereal* derived genes *Lr*26/*Sr*31/*Yr*9/*Pm*8. The gene *Lr26* is ineffective against more than 12 Indian leaf rust races and *Yr9* against 46S119 and 78S84 races of stripe rust.

The alien segment carrying linked genes *Lr*24/*Sr*24 does not impose yield penalty as several cultivars carrying *Lr*24 have been released for cultivation in India [12]. The resistance conferred by *Aegilops speltoides* derived gene *Lr*28 remained effective against Indian leaf rust races for a long period of time [13]. A cultivar MACS 6145 possessing *Lr*28 was deployed in 2006 [14]. Recently a virulence of the race 77-10 (377R60-1) has been reported against *Lr*28 [15] in India, though gene has not been deployed on a large area. Pyramiding of these two genes is expected to provide resistance against all the Indian races of leaf rust in the genetic background of HD2687. Stripe rust resistance gene *Yr15* is involved in race specific all stage resistance worldwide [5, 16]. Therefore, the present study was aimed of combining two leaf rust resistance genes, *Lr*24*, Lr*28 with stripe rust resistance gene *Yr*15 in the background of wheat variety HD2687, which is highly susceptible to rusts, to enhance durability of leaf and stripe rusts resistance.

Materials and methods

The plant material consisted a rust susceptible recipient variety HD2687 and resistance donors, TR380-14*7/3Ag#14, CS2A/2M#4/2 and Avocet*6/*Yr15* carrying *Lr24*, *Lr28* and *Yr15* genes, respectively. The bread wheat genotype, HD2687, a high yielding bread wheat genotype with potential yields of 6t/ha under high fertility and irrigated conditions has been derived from the cross CPAN2009 x HD2329. Advance backcross lines HD2687*3//TR380-14*7/3Ag#14 carrying *Lr24* (hitherto referred as HD2687*^{3}/*Lr*24), HD2687*3//CS2A/2M#4/2 carrying *Lr28* (hitherto referred as HD2687*^{3}*Lr*28) and HD2687/Avocet*Yr15* (F_1) carrying *Yr15* were obtained from the Division of Genetics, Indian Agricultural Research Institute, New Delhi. Backcross line, HD2687*3/*Lr24* was crossed with HD2687/Avocet*Yr15* (F_1) during *kharif* 2006 in off season nursery at IARI, RS, Wellington, Nilgiris to get BC_3F_1 of gene combination (*Lr24*+*Yr15*). Similarly HD2687*3/*Lr28* was also crossed with HD2687/Avocet/*Yr15* (F_1) to get BC_3F_1 of *Lr28*+*Yr15* gene combination. Plants looking similar to recipient genotype HD2687 were intercrossed during *kharif* 2008 in phytotron at IARI, New Delhi to combine three genes (HD2687*4/*Lr24*/Avocet*Yr*15(F_1)//HD2687*4/*Lr*28/Avocet*Yr*15(F_1)). BC_4F_1 plants carrying different gene combinations were selfed to get BC_4F_2 generation of pyramided lines. The desired crosses were made and alternate segregating generations were grown in off season at IARI Regional Station Wellington, the Nilgiris. Normal agronomical practices were followed for raising the crop. Pure rust inoculum of the race 77-5 (121R63-1) of leaf rust and two races of stripe rust 46S119 and 78S84, were obtained from DWR, Regional Station, Flowerdale, Shimla.

Seedling test

About 8-10 days old seedlings (DC11 = second leaf just visible [17]) were sprayed with leaf rust inoculum in the evening hours under glass house conditions. The inoculum was multiplied on Agra local. Prior to inoculation the plants were sprayed with water to provide a uniform layer of moisture on the leaf surface. The BC_4 progenies of HD2687 carrying *Lr24* and *Lr28* and donor Avocet/*Yr15* were incubated for 36 h in humid glass chambers at a temperature of 20±2^{o}C and more than 85% relative humidity. The disease reactions were recorded 12-14 days after inoculation using score method described by Stakmen *et al.* [18].

Adult plant resistance

Spreader rows were inoculated at boot leaf stage of plant growth [17] with urediospore suspension of a leaf rust pathotypes 77-5 and stripe rust pathotypes 46S119 and 78S84 in water using hypodermal syringe. Three to four tillers of spreader rows were inoculated with inoculum suspension at a distance of one meter. Adequate irrigation was given to provide sufficient humidity in the field for uniform spread of rust. The adult plant reactions were recorded by combining severity (percent infection) and host responses (type of infection). Rust severity was recorded according to the modified Cobb's scale described by Peterson *et al*. [19] and was estimated on the basis of percentage area covered with pustules.

Molecular markers for the leaf rust and stripe rust resistance genes

Before utilizing the donors for pyramiding the rust resistance genes in the genetic background of HD2687 molecular validation was carried out with the available known molecular markers to confirm the presence of selected genes for both leaf rust and stripe rust resistance. Characteristic features of molecular markers linked to the leaf rust resistance genes *Lr*24, *Lr*28 and stripe rust resistance gene *Yr*15 are presented in Table 1. Genomic DNA was isolated from either 7-10 day glasshouse grown seedlings or 3-4 week old field grown plants. DNA was isolated using modified CTAB method [20]. The amplified PCR products of SCAR markers and SSR markers were subsequently resolved on 2 % (W/V) agarose gel and 3.5% metaphor gel in 1X TBE buffer.

Results and discussion

Phenotypic reaction against rusts in the donors and recipient plant

The recipient genotype HD2687 and advance backcross lines HD2687*4//TR380-14*7/3Ag#14 carrying leaf rust resistance gene *Lr24*, HD2687*4//CS2A/2M#4/2 carrying *Lr28*, their donors TR380-14*7/3Ag#14, CS2A/2M#4/2 and Avocet*5/*Yr15* carrying *Yr15* gene were subjected to seedling and adult plant resistance test against most virulent and prevalent race 77-5 of *P. triticina* and 46S119 and 78S84 races of *P. striiformis* (Table 2). The seedling reaction against

Table 1. Details of markers used for the selection of *Lr*24, *Lr*28 and *Yr*15 gene

Genes	Molecular marker	Primer sequence (5'——3')	Product size (bp)	AT (°C)	Reference
Lr24	$SCS1302_{607}$ (SCAR)	F: CGC AGG TTC CAA TAC TTT TC R: CGC AGG TTC TAC CTA ATG CAA	607	60	[21]
Lr28	$SCS421_{570}$ (SCAR)	F: ACA AGG TAA GTC TCC AAC CA R: AGT CGA CCG AGA TTT TAA CC	570	60	[22]
Yr15	Xgwm273 (SSR)	F: ATT GGA CGG ACA GAT GCT TT R: AGC AGT GAG GAA GGG GAT C	165	55	[23]

Table 2. Rust reaction of parental lines at seedling and adult plant stages under artificial epiphytotic conditions

Parental lines	Reaction to			
	Leaf rust		Stripe rust	
	Seedling stage	Adult plant stage	Seedling stage	Adult plant stage
HD2687	3^+	70 S	3^+	70 S
Avocet*5/*Yr15*	3	40S	;=	TR
HD2687*4//CS2A/2M#4/2	0;	F	3	80 S
CS2A/2M#4/2	0;	F	3	80 S
HD2687*4//TR380-14*7/3Ag#14	0;	F	3	80 S
TR380-14*7/3Ag#14	3	5R	3	80 S

R= Resistant (hypersensitive flecks and small uredia with necrosis); S= Susceptible (Large uredia with or without necrosis or chlorosis); F= No infection, T- Traces

leaf rust pathotype 77-5 revealed a high level of resistance in HD2687*4//TR380-14*7/3Ag#14, TR380-14*7/3Ag#14 and HD2687*4//CS2A/2M#4/2, CS2A/2M#4/2 exhibiting infection type (IT) '0;' whereas the recipient genotype HD2687 exhibited susceptible reaction with the IT of 3^+ (Table 2). The Avocet*5/*Yr15* screened against stripe rust pathotypes 46S119 and 78S84 also exhibited high level of seedling resistance with IT ;=. The recipient HD2687 showed susceptibility with IT of 3^+. Both the backcross lines HD2687*4//TR380-14*7/3Ag#14 and HD2687*4//CS2A/2M#4/2 showed high degree of resistance against tested pathotypes of leaf rust, as against the recipient variety HD2687 which exhibited high susceptibility (70S) to both the rusts.

Molecular validation and marker assisted selection for Lr24, Lr28 and Yr15 genes in the backcross derived lines

The SCAR marker $SCS1302_{607}$ linked to the gene *Lr*24 [21] for leaf rust resistance was screened for polymorphism in the recipient genotype, HD2687 backcross line, HD2687*4/*Lr*24 stock carrying *Lr24,* TR380-14*7/3Ag#14, HW1042, HD2329+*Lr24* and control, Agra Local. The SCAR marker revealed the polymorphism by amplifying the *Lr*24 specific marker fragment of 607 bp size in the source genotypes (donor), TR380-14*7/3Ag#14, HW1042, HD2329+*Lr24* and the backcross line HD2687*4/*Lr*24 whereas the marker was absent in the recipient parent HD2687 as well as in control genotype Agra Local. Similarly the SCAR marker $SCS421_{570}$ linked to the leaf rust resistance gene *Lr28* [22] was validated in the genotypes, namely, HD2687, CS2A/2M#4/2, HD2877, HD2687*4/*Lr*28 and Agra Local. The marker revealed the polymorphism by amplifying the *Lr28* specific marker fragment of 570 bp size in the donor genotype, CS2A/2M#4/2 and backcross line HD2687*4/*Lr*28. The SSR marker Xgwm273 linked to the stripe rust resistance gene *Yr*15 [23] was validated in some known donors of the gene. The marker showed polymorphism by amplifying critical fragment of 165 bp in the *Yr*15 carrying genotypes such as, Sunstar*6/C80-1//V763-2312, V763-2312, and Avocet*5/*Yr*15 but not in the genotypes HD2687, Agra Local, HD2329 which were non-carriers of the *Yr*15 gene.

Advance backcross lines, HD2687*3/*Lr24* and HD2687*3/*Lr28* were crossed with HD2687/Avocet*Yr15* (F_1) to get BC_3F_1 of two gene combinations. All the BC_3F_1 plants were raised and tagged carefully. From each plant two to three leaves were collected for DNA isolation. The plants were subjected to molecular marker $SCS1302_{607,}$ $SCS421_{570}$ and Xgwm273 linked to the resistance genes *Lr24*, *Lr28* and *Yr15* respectively for identifying the presence of genes in combination of *Lr24+Yr15* and *Lr28+Yr15*. Out of 31 BC_3F_1 plants, five plants were identified carrying the combination of *Lr24+Yr15* whereas, 8 plants possessing *Lr28+Yr15* were identified from a population of 46 plants in BC_3F_1 population. Phenotypic selection at each backcross and selfing generation was conducted to eliminate plants with linkage drag traits such as tall plant type and late flowering. Plants looking similar to recipient genotype HD2687 were intercrossed to combine three genes, viz. *Lr24, Lr28* and *Yr15*. A total of 117 plants were obtained from BC_4F_1 generation. Out of 117 plants, 52 were identified having gene combination *Lr*24+*Lr*28. These 52 plants identified to carry the *Lr24* or *Lr28* genes with the help of SCAR marker analysis were subjected to molecular analysis for the presence of *Yr15* gene for stripe rust resistance. Of these, 12 plants were found carrying all the three genes, *Lr24+Lr28+Yr15*. Selfing of all the plants which carry *Lr24+Lr28* or *Lr24+Lr28+Yr15* gene combination was done to get BC_4F_2 pyramided lines. Out of 77 BC_4F_2 plants, eight plants with *Lr24+Lr28+Yr15*, 28 plants with *Lr24+Lr28*, 7 plants with *Lr24+Yr15* and 6 plants with *Lr28+Yr15* gene combinations were identified with the help of validated molecular markers (Fig. 1. a b c). All the pyramided lines were selected on the basis of dual selection procedure of HD2687 phenotype and foreground selection using *Lr*24, *Lr*28 and *Yr*15 gene specific DNA markers.

Agronomic performance of the pyramided lines

Agronomic data to compare the pyramided lines with the recipient genotype were also collected. The bar diagram represent comparative difference between different combinations of pyramided lines in BC_4F_2 generation (Fig. 2). The data indicated that Plant height, spike length, number of spikelet/spike and 1000 grain weight was found to be higher in HD2687. Number of tillers was higher in plants containing *Lr24/Yr15* than HD2687. Number of grains per spike was found to be higher in HD2687/*Lr24+Lr28* than the recurring parents. Pyramided line of HD2687 with *Lr*24, *Lr*28 and *Yr*15 was backcrossed with HD2687 to improve the recovery of genotype HD2687. Table 3 represents the descriptive statics of recipient parent HD2687 and three genes containing pyramided line. Paired *t* test was performed to see the significance of pyramided lines over HD2687 (Table 4) using SPSS software.

Table 3. Descriptive statistics of recipient parent HD2687 and BC_4F_2 pyramided lines

	HD2687						HD2687 (*Lr*24+*Lr*28+*Yr*15)					
	PH	TN	SL	SPN	GN	TKW	PH	TN	SL	SPN	GN	TKW
Mean	89.98	8.47	12.73	21.67	59.67	36.69	86.09	10.00	11.22	20.78	60.1	32.36
S.E.	0.380	0.515	0.24	0.32	1.09	0.58	0.54	0.47	0.39	0.52	0.61	0.57
Std. Dev.	1.47	1.99	0.96	1.23	4.24	2.24	1.62	1.42	1.19	1.56	1.83	1.69
Variance	2.16	3.98	0.93	1.52	17.95	5.02	2.63	2.00	1.41	2.45	3.36	2.88
Min	88	6	11	19	50	34	83	8	9	19	57	30
Max	93	12	15	23	66	40	89	12	13	23	63	36
t-value	237.02	16.43	51.21	67.98	54.54	63.43	141.63	19.65	24.97	35.16	89.28	50.41

PH= Plant height, TN= Number of tillers, SL= Spike length, SPN= Spikelet number, GN= grains per spike, TGW=1000 grain weight

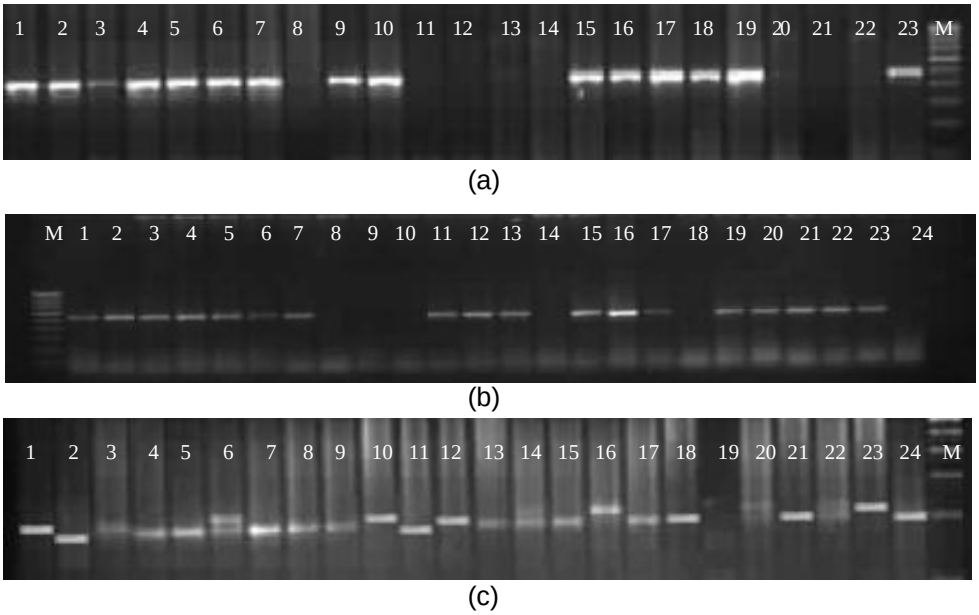

Fig. 1. Screening of rust resistance/susceptible individuals in BC_4F_2 generation with SCAR markers $SCS1302_{607}$ for *Lr*24, $SCS421_{570}$ for *Lr*28 and SSR marker Xgwm273 for *Yr*15 gene. (M= 100bp molecular weight marker, (a) Lane 1-23= Presence/absence of the linked marker fragment for *Lr24* gene, (b) Lane 1-24= Presence/ absence of the linked marker fragment for *Lr28* gene, (c) Lane 1-24= Presence/absence of linked marker in for *Yr15* gene); Indicating the plants which contain three gene combinations *Lr24, Lr28* and *Yr15*

Plant height, spike length and 1000 grain weight was significant giving two tailed *p* value less than 0.05 and other phenotypic characters were non significant as they were giving higher *p* value.

The intensive agricultural management practices associated with high yielding varieties have accentuated many disease problems due to favorable micro-climatic conditions. In such situations, the agronomical superiority of wheat varieties and effective disease management gains importance for stable and sustainable production. It is believed that, in wheat, certain gene combinations give better and long lasting resistance to rust diseases than given by any of the genes individually [24]. One of the main disadvantages in using single gene resistance is that because of rapid changes in predominant rust pathogen races (pathotypes) in nature, single-gene resistance in a cultivar may become ineffective soon after it is released. In this context, gene pyramiding of effective rust resistance genes is probably the faster strategy to develop rust resistant wheat cultivars. Gene pyramiding can be greatly facilitated with associated markers through marker assisted selection programs

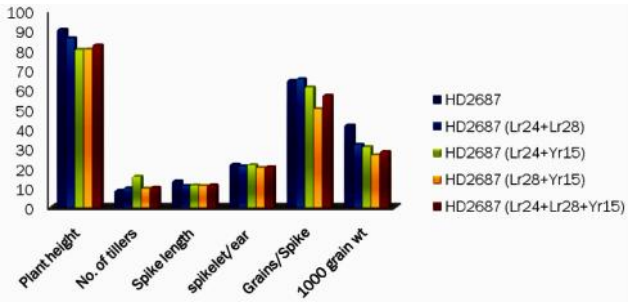

Fig. 2. Comparative difference among the pyramided lines of BC_4F_2 generation

(MAS), this is particularly true in the field of wheat breeding for leaf rust resistance where PCR-based markers are already available for almost half of the 60 or more designated resistance genes and alleles [7]. For example, MAS has been used successfully to introgress into adapted germplasms from Hungary gene combinations *Lr*9 + *Lr*24, *Lr*9 + *Lr*25 and *Lr*9 + *Lr*29 [25].

In the present study two dominant markers for leaf rust resistance genes *Lr24* and *Lr28* and a co-dominant marker for stripe rust resistance gene *Yr15* were employed to screen the backcross populations. It highlights the role of DNA markers linked to resistance genes, minimizing the need for epiphytotic methods in selecting for resistance against the diseases. Different gene combinations were obtained in BC_4F_2 generation with the help of molecular markers linked to the gene(s). Due to presence of two or three gene combination it was possible that phenotyping for the disease may be inconsistence, because of disease escaped susceptible plants could not develop infection due to micro environmental variation. In such situation, the molecular markers were ideal to detect the resistance genes in plants which were otherwise morphologically desirable. Under rust free conditions molecular markers are able to identify the plants carrying different gene combinations.

There are several studies where marker assisted pyramiding has been done to improve durability of rust resistance in wheat. Bhawar *et al*. [9] has pyramided leaf rust resistance gene *Lr*19 and *Lr*28 in F_2 population and used around 602 SSR markers distributed uniformly in all chromosomes for background analysis of recurrent parent. Similarly, genes, *Lr*24, *Lr*28 and *Yr*15 have been combined in the background of wheat variety HD2877 using molecular markers to validate the presence of resistance genes and selected pyramided lines in BC_2F_2 generation [8]. The successful marker assisted pyramiding of disease resistance genes is also reported in wheat with respect to three leaf rust resistance genes *Lr*13, *Lr*34 and *Lr*37 [26] and three powdery mildew genes *Pm3*, *Pm4a* and *Pm21* [27]. A similar study was carried out by Suh *et al*. [11] for the development of breeding lines with pyramided resistance genes *Xa*4, *Xa*5 and *Xa*21 by foreground and phenotypic selection in rice. Accumulating major genes for resistance is laborious, time consuming and very difficult when two or more resistance genes are pyramided into an elite cultivar. However marker assisted backcrossing with accurate phenotypic selection is the most effective method for selective transfer or pyramiding of resistance genes into a

Table 4. Paired *t* test between the groups HD2687 and BC_4F_2 pyramided lines

Traits combinations		Mean	SD	SE(±) of mean	95% confidence interval of the difference		*t* value	Significance (2-tailed *p* value)
					Lower	Upper		
Pair 1	PH - PH_PL	3.77	2.61	0.92	1.59	5.95	4.095	0.005
Pair 2	TN - TN_PL	-1.50	2.21	0.78	-3.34	0.34	-1.93	0.096
Pair 3	SL - SL_PL	1.57	1.69	0.59	0.17	2.98	2.65	0.033
Pair 4	SPN - SPN_PL	1.25	1.83	0.65	-0.28	2.78	1.93	0.095
Pair 5	GN - GN_PL	-1.37	5.15	1.82	-5.68	2.94	-0.76	0.475
Pair 6	TGW - TGW_PL	3.84	3.32	1.17	1.06	6.61	3.27	0.014

PH= Plant height of HD2687, PH_PL= Plant height of pyramided line, TN= Number of tillers of HD2687, TN_PL= Number of tillers of pyramided line, SL= Spike length of HD2687, SL_PL= spike length of pyramided line, SPN= Spikelet number of HD2687, SPN_PL= Spikelet number of pyramided line, GN= Grains per spike of HD2687, GN_PL= Grains per spike of pyramided line, TGW= 1000 grain weight of HD2687, TGW_PL= 1000 grain weight of pyramided line, SD=Standard deviation, SE=Standard Error

cultivar free from linkage drag, eventually restoring the recurrent parent genotype.

In our study phenotypic selection at each backcross and selfing generation was conducted to eliminate plants with linkage drag traits such as tall plant type, short spikes length, less tillers and late flowering. Agronomic data to compare the pyramided lines with the recipient genotype were also generated. The data indicated that plant height, number of tillers, spike length, number of spikelets/ear and 1000 grain weight was found to be higher in the recurrent parent HD2687 in BC_4F_1 generation whereas, in BC_4F_2 generation pyramided lines were more similar to recurrent parent HD2687 indicating good recovery of genetic background. Paired *t* test within group of HD2687 and pyramided lines show that plant height, spike length and 1000 grain weight were significant. However, the agronomic data indicated that the resistance genes have impeded grains per spike and number of tillers. The lines obtained with different combination of rust resistance genes in HD2687 background can be used for gene deployment and molecular studies.

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