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Characterization of wheat (*Triticum aestivum* L.) mutants for improved agronomic traits and disease resistance

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Induced mutation holds promise as a strategy for creating new variations. In this study, we analysed mutant lines of cultivar HD 3086 selected for its extensive cultivation in India, at both morphological and molecular levels. A total of 150 M_4 mutant lines exhibited improved yield attributes, enhanced disease resistance and other agronomically important traits. Evaluation of mutant lines against leaf rust race 77-9 and stripe rust race 238S119 identified four and six highly resistant lines, respectively. SNP genotyping of rust-resistant mutants using 35K Axiom array revealed polymorphism rates ranging from 7.1 to 11.9% compared to the parent line, HD3086. Several induced point mutations were observed that may underlie key genes involved in disease resistance. The identified mutant lines, particularly the rust-resistant ones, have strong potential as valuable donors in breeding programs and as a resource for fundamental genetic studies in the future.

Keywords: biotic stress, leaf rust, mutation, stripe rust, *Triticum aestivum*

Wheat (*Triticum* spp.) is one of the major cereal crops that was domesticated in the Middle East more than 10,000 years ago. It is the third most cultivated crop, with a gross annual production of ~798 million tons in 2024 (http://faostat.org). In India, wheat is cultivated across an area of 32.8 million hectares, producing 117.9 million tons with a productivity of 3.59 tons per hectare in 2024-25, making it the second-most significant crop after rice (https://upag.gov.in). However, due to changes in climatic conditions, wheat production is negatively impacted by various biotic and abiotic stresses[1].

Among the various biotic stresses affecting the wheat crop, rust diseases are the most significant in terms of the production losses[2]. All three rusts, namely leaf rust (*Puccinia triticina*), stem rust (*P. graminis*), and stripe rust (*P. striiformis*), are known to cause substantial damage across different agro-ecological zones[3]. In particular, stripe rust predominates in the Northwestern Plain zone, stem rust in the Central and peninsular zones, while leaf rust is widespread across all these regions. In Southeast Asia, stripe and leaf rust have caused major losses in wheat harvests in recent years[4]. Only a few resistance genes are effective at preventing the emergence of new races[4]. Identifying lines resistant to the newer races of leaf and stripe rust will enhance our preparedness to combat these diseases and provide significant benefits to breeding programs. Furthermore, breeding for rust resistance reduces the reliance on chemical pesticides and enhances yield stability.

Numerous approaches have been employed to develop tolerant lines, among which mutation breeding stands out as a valuable method for generating new favourable alleles in crops. Induced mutation holds promise as a strategy for creating new varieties with improved agronomic traits, including enhanced tolerance to biotic and abiotic stresses and bio-fortification[5,6]. Both physical (radiations) and chemical mutagens, such as EMS[7] have been widely used in crop improvement in the past[8-11]. These mutagens can improve either a single trait or an entire line and can introduce novel traits into existing germplasm. To date, 3402 mutant varieties have been produced across various crops (https://mvd.iaea.org/), of which_265 are mutant wheat varieties. In wheat, mutant varieties have been developed to address challenges such as yield improvement, disease resistance, increased protein content, and enhanced bread and chapatti quality[12-14].

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In this study, a widely adopted wheat cultivar, HD3086, extensively cultivated across timely sown irrigated conditions in the Northern Plain Zone of India, was selected[15]. It has recently shown increased susceptibility to major pathotypes of *P. triticina* and *P. striiformis*, as well as heightened temperatures. The present study, therefore, focused on characterising mutagenised HD3086 lines for improved traits capable of thriving in stressful environments. The studied plants exhibited a variety of mutant phenotypes, showcasing variations in morphological and agronomic characteristics. A comprehensive analysis of these variations and the characterization of various traits aided in the identification of novel mutant lines.

Materials and Methods

The seeds (~800) of a widely known wheat cultivar, HD 3086, were irradiated with gamma radiation at a dosage of 250 Gy[16]. For the M_1 generation, seeds were sown in a completely randomised block design with 8 seeds per meter row, with rows spaced 23 cm apart. Single spikes were collected from each plant to produce the M_2 generation, as illustrated in Fig. 1. A total of 2398 mutants in the M_2 generation obtained through a meticulous single-seed descent approach (by planting seeds of each spike into a single row) were evaluated for alteration in various phenotypic characters, including plant height, spike characteristics, disease resistance, and agronomic traits. To ensure the highest quality, the seeds were harvested from a single plant showing the best phenotype for each M_2 mutant. The

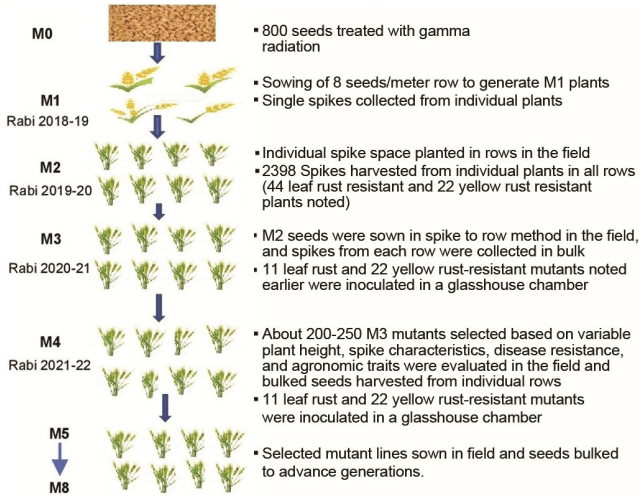

Fig. 1 — Schematic plan for advancement of mutant lines.

lines were progressively advanced to the M_5 generation, with seeds bulked from each row. The mutant lines that displayed variability in key attributes such as plant height, awn character, rust resistance, heading variation, yield, and related traits were selected, excluding those exhibiting stunted growth, pollen sterility, and poor germination.

Phenotyping

The M_2 mutant lines were evaluated for both stripe and leaf rust resistance naturally under field conditions during the 2019-20 crop season. To ensure there is no disease escape in the field, the selected resistant lines were screened for leaf rust resistance race 77-9 under artificial inoculation conditions for two years (2020-21 and 2021-22) at IARI Regional Station, Flowerdale, Shimla. Particularly, 77-9 is a predominant race of leaf rust pathogen persisting for the last five years in the Northern plains of India. Furthermore, the selected lines were additionally assessed under artificial inoculation conditions with the race 238S119 of the stripe rust pathogen for three consecutive years (2020-21, 2021-22 and 2022-23). 238S119 is another predominant race for stripe rust in recent years.

Fifteen seedlings were evaluated per mutant line for both leaf and stripe rust races in aluminium trays. Briefly, ten-day-old seedlings with completely opened primary leaves were inoculated with leaf and stripe rust pathotypes 77-9 and 238S119, respectively. Inoculation mixture was prepared by adding urediospores in water along with a drop of Tween 20 to attain affinity and uniform spread of the urediospores on the leaf surface. After inoculation, the plants were transferred to a dew chamber for 48 h and finally transferred to a glasshouse bench maintained at 15±2 °C for stripe rust and 20±2 °C for leaf rust. During the entire process of inoculation, aseptic conditions were maintained in the glasshouse to avoid contamination. The response of mutant lines for leaf rust and stripe rust pathogen races was recorded using a standard scoring scale of 0-4, as established by Stackman *et al*. (1962)[17]. The evaluation was based on uredinia size and the presence or absence of necrotic (N) or chlorotic regions (C). Agra Local served as a susceptible disease check for all these evaluations. The resistant plants observed in the segregating mutants were carefully transferred from trays to pots to obtain seeds for the next generation.

Infection responses were recorded as follows: 0 for immune reactions with no visible uredia, ; for absence

of uredia but with hypersensitive flecks, 1 for resistant reactions characterised by small uredia with necrotic areas, 2 for moderately resistant reactions with small uredia surrounded by either chlorosis (C) or necrosis (N), 3 for moderately susceptible/ resistant reactions with medium-sized uredia associated with chlorosis, and 4 for highly susceptible reactions with large uredia and chlorosis. To perform analysis of variance (ANOVA) and estimate critical differences (CD) among lines across different generations, Stackman's 0-4 scoring scale was converted into a linear quantitative scale ranging from 0 to 9[18]. Infection types 0, 1-, 1, 1+, 2-, 2, 2+, 3-, 3, and 3+ were coded as 0, 1, 2, 3, 4, 5, 6, 7, 8, and 9, respectively. The hypersensitive fleck (;) symbol was coded as 0, and infection type 4 was coded as 9. Descriptors indicating necrosis (N) and chlorosis (C) were ignored during the conversion.

SNP Genotyping

The genomic DNA of a selected subset of lines, focusing on rust resistance, was isolated from leaves by using the protocol of Prabhu *et al*. (1998)[19]. Four M_4 mutant lines selected for each leaf rust and stripe rust resistance, and the wild type, were subjected to SNP genotyping via a 35K SNP chip, specifically the Wheat Axiom Breeders array (Affymetrix, Santa Clara, CA, United States). To identify single-nucleotide changes, the selected mutants were compared to the wild-type parent at distinct chromosomal loci. The SNP filtering was carried out for missing data and monomorphic alleles in mutant lines and the wild-type parent. The physical locations and the mapped genes for filtered SNPs were obtained from the Ensembl database (www.ensembl/triticum). To determine the putative function, the genes associated with the SNPs identified in the mutant lines were searched for their expression in the Wheat Expression Browser by expVIP[20,21] (http://www.rust-expression.com/) and were noted down in the transcript per million (TPM).

Results

Identification of mutants carrying important traits

In the M_2 generation, a substantial level of variation (297/2398; 12.4%) in morphological characteristics was observed compared to the control cultivar HD3086. The observed variation encompassed traits such as plant height, glaucousness, days to heading, maturity, and the presence or absence of awns (Fig. 2A-J). Among the mutants, 20 lines exhibited variations in leaf morphology, including upright leaf angle, broad lamina, and curled

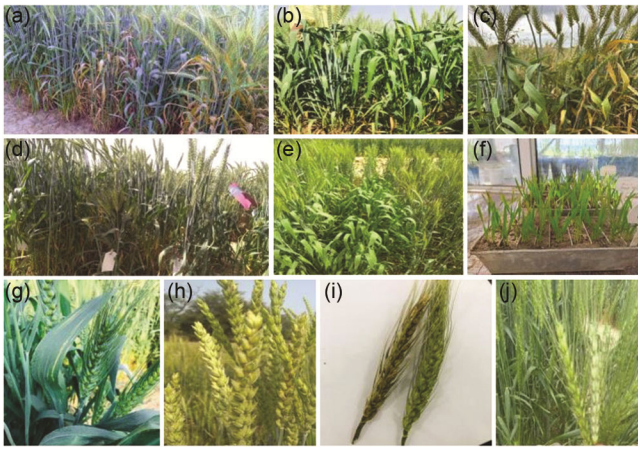

Fig. 2 —Mutants identified for morphological traits (a) stripe rust resistance in the field; (b) non-glaucous and glaucous; (c) leaf rust resistant mutant in the field; (d) dwarf; (e) late heading; (f) stripe rust resistance during seedling inoculation; (g-h) spike characteristics *viz*, clubbed spikes, reduced awns; (i) pseudoblack spike; (j) spike glaucousness.

leaves. In addition, 55 mutant lines displayed diverse spike characteristics, such as compact or clubbed spikes, variation in spike length, number of spikelets, grain weight, and other related traits. To identify stable mutations, the mutant lines were screened across the M_3 and M_4 generations. In the overall evaluation of the M_4 generation, 8 mutant lines were taller than HD3086, while 3 lines were dwarf in comparison. Furthermore, 22 lines exhibited varying levels of resistance to stripe rust, 11 to leaf rust, 14 showed reduced awn length, 10 displayed a non-glaucous phenotype, and 18 exhibited variations in heading time. Numerous lines also showed variability in leaf architecture, spike characteristics, and yield-related traits (Table 1). Notably, the mutant lines with reduced awns were taller than the parent HD3086 but were susceptible to rust diseases.

Screening mutant lines for rust resistance

Among the M_2 population, 44 mutant lines exhibited differential resistance to leaf rust under field conditions. Upon further evaluation through artificial inoculation with leaf rust race 77-9, 11 mutants showed a clear and consistent response to leaf rust, while 33 mutants segregated for leaf rust resistance (Table 2). Of the 11 stable lines, seven exhibited moderate to high resistance, whereas four showed moderate to high susceptibility. Notably, the mutant lines HD 3086 5-01, HD 3086 35-06, HD 3086 49-01 and HD 3086 291-02 consistently displayed high levels of resistance over two consecutive generations (M_3 & M_4). Seeds from resistant plants of these mutants were bulked for large-scale multiplication trials.

A total of 22 M_2 mutant lines demonstrated resistance to stripe rust under field conditions. These resistant lines were subsequently evaluated under artificial inoculation with the pure stripe rust pathogen race 238S119 across M_3, M_4 & M_5 generations. Varying levels of resistance were observed among the mutants: six lines were highly resistant, six exhibited moderate resistance, and nine were moderately susceptible (Table 3). The wild-type HD 3086 showed susceptibility to the 238S119 race, while HD 3086 09-08, HD 3086 32-08, HD 3086 115-07, HD 3086 170-2, HD 3086 263-02 and HD3086 331-03 exhibited a high level of resistance. The analysis of variance unveiled significant differences between mutant lines for both leaf and stripe rust resistance (Table 4).

Table 1 — Mutants identified for various phenotypic attributes in different generations

Trait	Phenotype	Number of M_3 mutants	Number of M_4 mutants
Plant Height	Dwarf	3	3
	Tall	13	8
Heading	Early	5	7
	Late	11	11
Spike	Compact	1	1
	Reduced awns	14	14
	More spikelets	11	9
	High grain wt	9	15
Glumes	Dark colour	7	5
	Pseudoblack	5	2
Leaf morphology	Broad Shape	2	1
	curl	16	6
Stem appearance	Non-Glaucous	14	10
Rust disease	Highly susceptible	6	14
	Moderate/highly resistant	66	19
Miscellaneous	crooked spikes, yellow spikes, reduced spikelets etc.	21	25
Total		204	150

Molecular characterization of selected mutant lines

The mutant lines exhibiting leaf rust resistance had 6.8 to 7.1% of polymorphism compared to the HD3086 parent, whereas the mutant lines resistant to stripe rust exhibited higher polymorphism, ranging from 7.1 to 11.9% of polymorphism as detected using the Axiom 35K SNP array**.** The proportion of SNPs was higher for chromosomes 1, 2, 5, and 7 in both leaf and stripe rust resistant lines, particularly when considering only SNPs at homozygous loci and excluding heterozygous ones (Fig. 3A & B). The average frequencies of nucleotide substitutions were relatively high in both leaf and stripe rust resistant lines, with transitions (purine to purine and pyrimidine to pyrimidine) occurring at approximately 361 and 437, respectively, while transversions (purine to pyrimidine and vice versa) occurred at approximately 168 and 193 nucleotides, respectively (Fig. 3C).

Table 2 — Response of mutant lines in different generations to leaf rust race 77-9 during artificial inoculation based on a 0-4 scoring scale. Figures in parentheses are conversion values on a 0-9 scale.

Genotypes	Disease Score		Response of mutants
	M_3	M_4	
HD 3086 05-01	0; (0)	0 (0)	Highly resistant
HD 3086 291-02	1N (2)	1+ (3)	Highly resistant
HD 3086 35-06	0 (0)	1- (1)	Highly resistant
HD 3086 83-06	2N (5)	2+ (6)	Moderately resistant
HD 3086 369-06	2C (5)	2 (5)	Moderately resistant
HD 3086 49-06	3-N (7)	3+ (9)	Moderately Susceptible
HD 3086 49-01	0; (0)	1- (1)	Highly resistant
HD 3086 306-04	3C (8)	3 (8)	Moderately Susceptible
HD 3086 09-08	2-N (4)	1- (1)	Moderately resistant
HD 3086 89-4	2-N (4)	2+ (6)	Moderately Susceptible
HD 3086 54-3	3-C (7)	3+ (9)	Moderately Susceptible
HD3086	3+ (9)	3+ (9)	Highly susceptible
Agra local	3+ (9)	3+ (9)	Highly susceptible
CD (mean)	1.95	-	-

Stackman *et al* (1962) sacle of 0-4 converted to a quantitative scale of 0-9 (Zhang *et al*. 2023). 0-4 scale: 0 for immune with no visible uredia, ; for no uredia but hypersensitive flecks, 1 for resistant with small uredia with necrotic areas, 2 for moderately resistant with small uredia surrounded by either chlorosis (C) or necrosis (N), 3 for moderately susceptible/ resistant with medium-sized uredia associated with chlorosis, 4 for susceptible with large uredia with chlorosis. 0-9 scale: Infection types 0, 1- , 1, 1+ , 2- , 2, 2+ , 3- , 3, and 3+ are coded as 0, 1, 2, 3, 4, 5, 6, 7, 8, and 9, respectively. Hypersensitive fleck (;) is converted to 0 and 4 is converted to 9.

Table 3 — Response of mutant lines in different generations to stripe rust race 238S119 during artificial inoculation based on a 0-4 scoring scale. Figures in parentheses are conversion values on a 0-9 scale.

Genotypes	Disease Score			Response of mutants
	M_3	M_4	M_5	
HD 3086	3+ (9)	3+ (9)	3+ (9)	Highly susceptible
HD 3086 32-08	;1 (2)	0; (0)	1N (2)	Highly resistant
HD 3086 115-07	0; (0)	;1 (2)	1+ (3)	Highly resistant
HD 3086 170-02	0; (0)	0; (0)	0; (0)	Highly resistant
HD 3086 263-02	0; (0)	0; (0)	; N (0)	Highly resistant
HD 3086 5-01	2-N (4)	2-N (4)	0; (0)	Moderately resistant
HD 3086 09-08	0; (0)	;1 (2)	;1- (1)	Highly resistant
HD 3086 51-05	(1)	2-N (4)	(4)	Moderately resistant
HD 3086 54-05	0 (0)	2-N (4)	2N (5)	Moderately resistant
HD 3086 83-06	3+ (9)	3-N (7)	(7)	Moderately susceptible
HD 3086 85-07	3 (8)	3C (8)	2˙C (4)	Moderately susceptible
HD 3086 89-04	3+ (9)	3-C (7)	3-(7)	Moderately susceptible
HD 3086 91-03	3-C (7)	3C (8)	3 (8)	Moderately susceptible
HD 3086 184-02	0 (0)	2-N (4)	0; (0)	Moderately resistant
HD 3086 240-03	2-N (4)	3-N (7)	2-N (4)	Moderately susceptible
HD 3086 315-03	(7)	3N (8)	2N (5)	Moderately susceptible
HD 3086 330-05	(4)	3N (8)	3N (8)	Moderately susceptible
HD 3086 331-03	0; (0)	0; N (0)	0; (0)	Highly resistant
HD 3086 332-06	1+ (3)	2-N (4)	2-N (4)	Moderately resistant
HD 3086 343-01	(5)	(7)	3 (8)	Moderately susceptible
HD 3086 371-06	3+ (9)	3C (8)	3-C (7)	Moderately susceptible
HD 3086 421-04	0; (0)	2-N (4)	2N (5)	Moderately resistant
Agra local	3+ (9)	3+ (9)	3+ (9)	Highly susceptible
CD (mean)	2.651	-	-	-

Table 4 — Analysis of variance for leaf rust and yellow rust resistant wheat mutant lines

	Leaf rust				Stripe rust				
Source of Variation	DF	Sum of Squares	Mean Squares	F-Calculated	DF	Sum of Squares	Mean Squares	F-Calculated	Significance
Replication	1	0.038			2	20.609			
Treatment	12	283.462	23.622	29.959	22	602.435	27.383	10.626	< 0.001
Error	12	9.462	0.788		44	113.391	2.577		
Total	25	292.962			68	736.435			

In total, 221 genes (65%) in leaf rust resistant lines and 363 genes (67.1%) in the stripe rust resistant lines were associated with polymorphic SNPs. Of these, only 144 and 219 genes, respectively, had known protein functions. Interestingly, 114 characterized genes (31.4%) were shared between the leaf and stripe rust resistant lines (Fig. 3D). Among the genic variants, a large proportion of SNPs were located in untranslated regions (UTRs) and introns. In addition, several variants within coding regions resulted in synonymous and missense variations (Fig. 3E).

Discussion

Mutation breeding and plant mutagenesis are some of the most effective tools for augmenting genetic diversity in food crops and for uncovering their regulatory genes and molecular pathways. In the present study, we characterized gamma-ray-induced mutants of a high-yielding popular cultivar, HD3086, for altered traits of economic importance and disease resistance. Although a considerable number of mutations were evident in the M_2 generation, many of them were unstable and not observed in subsequent generations. Therefore, we focused our studies on the stable mutants in M_3, M_4 and M_5 generations, and their advancement for further identification of lines with major emphasis on disease resistance, which is one of the primary factors in developing an improved cultivar.

As the newer pathotype races continue to evolve, primarily in the context of rust diseases, it is essential to search for new genes and screen novel germplasm for the identification of resistant sources. Leaf rust

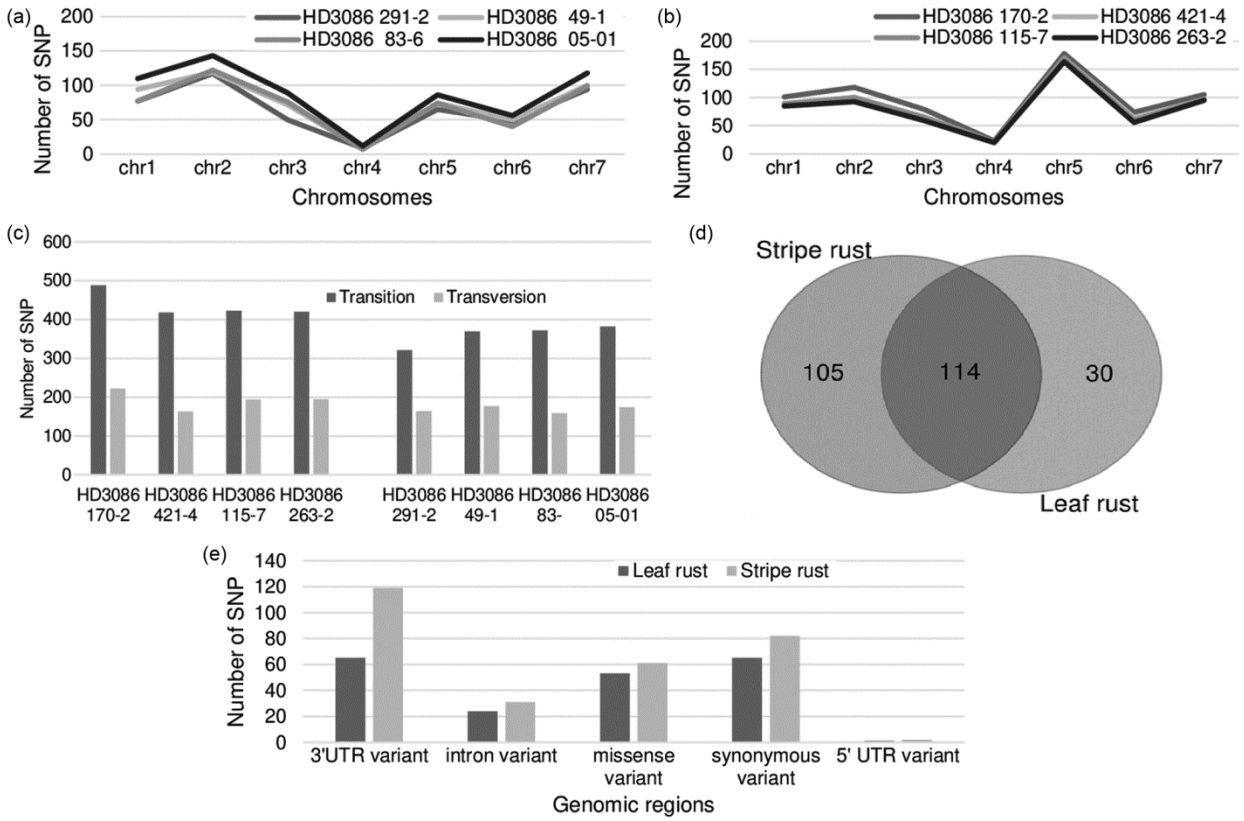

Fig. 3 — Localization of SNPs on seven chromosomes for, (a) leaf rust resistant; (b) and stripe rust resistant lines; (c) Number of SNPs undergoing transition and transversions in rust resistant lines; (d) shared genes between leaf and stripe rust resistant lines; (e) SNP variants belonging to different genomic regions.

pathotype, 77-9, has been prevalent since 2016, while the stripe rust pathotype, 238S119, has emerged as a major rust pathotype[4,22] in the Northern Plains zone since 2019. In view of this evolving scenario, the decade-established high-yielding cultivar HD3086 has become susceptible to these newer rust pathogen races. In this study, several improved variants of HD3086 were identified, including four leaf rust and six stripe rust resistant lines (as outlined in Tables 2 & 3). These lines hold great promise as suitable donors for crop improvement programs and as valuable materials for future gene expression studies. In plants, negative regulators of immune response function to prevent susceptibility. The cultivar HD3086 was susceptible to leaf rust race 77-9 and stripe rust race 238S119; however, the mutant lines developed resistance or complete immunity to these races, suggesting the presence of mutations in genomic regions that promote susceptibility in the parent line, HD3086. Host plants can actively facilitate susceptibility to pathogens. By studying isogenic lines that differ in their responses to specific pathogen races, as obtained in this study, genes involved in promoting disease susceptibility can be explored, and their mutant alleles can be identified for use in future breeding programs. In several crops, such susceptibility (S) genes have previously been inactivated through classical mutation breeding by identifying defective alleles within mutagenized plant populations, such as the *Enhanced Disease Resistance1 (EDR1)*[23] gene in barley.

Molecular characterization of germplasm holds significant promise for enhancing the efficiency of wheat breeding. Recent advances in genomics and molecular techniques such as exome capture sequencing, genotype-by sequencing, and single nucleotide polymorphism (SNP) analysis have been instrumental in the molecular characterization of mutations[7,24,25]. Gamma rays are known to induce small deletions and a higher frequency of single-base substitutions and deletions[25,26]. In the past, a substantial number of SNPs, indels, or deletions/insertions have been reported in mutant lines [8,11,27]. SNP genotyping of the selected mutants

for both leaf rust and stripe rust revealed ~7% to ~ 12 % of polymorphism with the wild type, signifying that these mutant lines differed from the wild type at single nucleotide positions at several loci across all chromosomes. Moreover, a considerable portion of these SNPs (144 in leaf rust & 219 in stripe rust resistant lines) were located within genic regions of chromosomes, suggesting that mutations occured within the transcribed regions of the genome (Table S1 and Fig. 3). Besides silent mutations and intron/UTR variants, numerous missense mutations may be explored for their role in rust resistance. Further, the genes associated with the altered SNPs, when searched for their presence in wheat rust expression (expVIP) databases, revealed 74 genes that were induced upon infection with stripe rust pathogen races and are summarized in Table S1. These genes majorly lie on 2A, 5A and 5B chromosomes, which may putatively carry the mutation for rust resistance. Subsequent Mutmap studies are being done in our lab to map the mutated resistant locus. The SNP genotyping results indicate that gamma irradiation induced several point mutations, some of which may be putatively linked to economically important genes, including rust resistance.

Conclusion

Mutagenesis in the wheat variety HD3086 resulted in significant genetic variability, affecting agronomic performance and rust disease resistance. Stable mutant lines identified in advanced generations showed enhanced resistance against leaf rust (race 77-9) and stripe rust (race 238S119). The mutant lines identified in this study can serve as valuable genetic resources for breeding programmes. Furthermore, these mutant lines hold potential for mapping genes associated with various traits, using mutmap or other advanced techniques in our future studies. The mutants serve as a valuable resource for both fundamental research and the generation of new varieties.

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Conflict of interest

The author(s) declare that they have no conflict of interest.

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