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ORIGINAL ARTICLE

Fine mapping of hybrid necrosis gene *Ne1* in common wheat (*Triticum aestivum* L.)

Yaoqi Si[1,2] · Shusong Zheng[1,2] · Jianqing Niu[1] · Shuiquan Tian[1,2] · Xiaoli Shi[1] · Yilin He[1,2] · Yiwen Li[1] · Hong-Qing Ling[1,2]

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Abstract

Key message **Hybrid necrosis gene *Ne1* was delimited into an approximate 4.06 Mb region on chromosome arm 5BL and an InDel marker that co-segregated with *Ne1* alleles was developed.**

Abstract Hybrid necrosis in wheat, characterized by progressive chlorosis and necrosis of plant leaves, tillers or whole plants in certain hybrids, is caused by complementary genes *Ne1* and *Ne2* located on chromosome arms 5BL and 2BS, respectively. Hybrid necrosis can be a barrier in combining desirable traits from various wheat genotypes. In this study, we fine mapped *Ne1* on chromosome arm 5BL, and delimited it to a 4.06 Mb region using large segregating recombinant inbred line families from cross 'Zhengnong 17' × 'Yangbaimai'. Genetic characterization confirmed that the *ne1* allele was closely associated with a 2.89 Mb deletion in Zhengnong 17. A tightly linked InDel marker, *5B-InDel385*, for *Ne1* was developed and was used to predict the presence of *Ne1* in a diverse panel of 501 common wheat accessions. Among those accessions, 122 (61%) of 200 landraces were predicted to carry the *Ne1* allele, whereas only 79 (26%) of 301 modern cultivars were predicted to carry *Ne1*. The significant decrease in *Ne1* frequency in modern cultivars indicated that the *Ne1* allele had been negatively selected in wheat breeding. This study provides a foundation for marker-assisted selection, gene cloning and functional studies of *Ne1* in wheat.

Introduction

Hybrid necrosis in wheat is characterized by premature senescence or death of leaves, tillers and even the whole plants in certain hybrids (Caldwell and Compton 1943; Hermsen 1963a). Hybrid necrosis can be lethal or semilethal resulting in gradual death of vegetative tissues or loss of seed productivity (Chu et al. 2006; Tsunewaki 1960). Thus, hybrid necrosis can be a barrier to gene transfer in breeding and can hinder the genetic improvement of wheat (Bizimungu et al. 1998; Galaiev 2016; Tomar et al. 1991).

Hybrid necrosis is caused by interaction of complementary dominant genes *Ne1* and *Ne2*, located in chromosome arms 5BL and 2BS, respectively (Chu et al. 2006; Kandel et al. 2017; Nishikawa et al. 1974; Pukhalskiy et al. 2018; Zeven 1972). Necrosis-inducing alleles of these genes are widely distributed in tetraploid and hexaploid wheat (Tsunewaki 1992; Vikas et al. 2013). Based on the relative strengths of necrosis symptoms, three dominant alleles ($Ne1^w$, $Ne1^m$, and $Ne1^s$) were identified at the *Ne1* locus, and five dominant alleles ($Ne2^w$, $Ne2^{wm}$, $Ne2^m$, $Ne2^{ms}$, and $Ne2^s$) were reported for the *Ne2* locus (Hermsen 1960, 1963a; Pukhalskiy et al. 2019). A wheat genotype carrying only the *Ne1* allele (*Ne1Ne1ne2ne2*) or the *Ne2* allele (*ne1ne1Ne2Ne2*), or neither (*ne1ne1ne2ne2*), has a normal green phenotype. When *Ne1* and *Ne2* alleles are both present (*Ne1-Ne2-*), hybrid necrosis appears, and the degree of necrosis is determined by the particular combination of alleles at the *Ne1* and *Ne2* loci (Hermsen 1963a). However, the molecular basis of wheat hybrid necrosis is still largely unknown.

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✉ Shusong Zheng
shusong@genetics.ac.cn

✉ Hong-Qing Ling
hqling@genetics.ac.cn

[1] The State Key Laboratory of Plant Cell and Chromosome Engineering, Institute of Genetics and Developmental Biology, the Innovative Academy of Seed Design, Chinese Academy of Sciences, Beijing 100101, China

[2] University of Chinese Academy of Sciences, Beijing 100049, China

Due to the widespread occurrence of *Ne1* and *Ne2* alleles in wheat accessions, it is useful to know which alleles might be present in order to avoid the occurrence of necrotic F_1 and thus to ensure useful progeny (Chu et al. 2006; Galaiev 2016). In the past, researchers relied on hybridizations with fixed testers containing genotype *ne1ne1Ne2*^{s}*Ne2*^{s} or *Ne1*^{s}*Ne1*^{s}*ne2ne2* to test if a genotype carried a particular necrosis allele (Hermsen 1963a). This approach is time-consuming, laborious, and limited in accuracy as the allele prediction is a qualitative assessment and the symptoms of hybrid necrosis are affected by degree of dominance, genetic background and environmental factors (Hermsen 1963a). Chu et al. (2006) mapped the hybrid necrosis loci *Ne1* and *Ne2* using SSR markers and demonstrated that markers *Xbarc74-5B* and *Xbarc55-2B* were linked to *Ne1* and *Ne2* at genetic distances of 2.0 cM and 3.2 cM, respectively. Due to the relatively loose genetic distances between the markers and *Ne* genes, incorrect predictions were likely and use of markers was adopted only in a limited way (e.g., Galaiev 2016; Kandel et al. 2017). Therefore, it is necessary to develop a more convenient and efficient molecular marker for prediction of *Ne* alleles in wheat germplasm.

Although hybrid necrosis was first reported in the 1940's (Caldwell and Compton 1943), *Ne* loci have not been fine-mapped or isolated. In this study, we developed a recombinant inbred line (RIL) population segregating at the *Ne* loci, and used selected segregating lines to fine map the *Ne1* gene. We developed an InDel marker that co-segregated with *Ne1* in a large mapping population. We also determined the distribution of *Ne1* in bread wheat accessions using the InDel marker.

Materials and methods

Plant materials

A RIL (F_8) population containing 188 lines was developed from an F_2 population derived from the cross 'Zhengnong 17' (ZN17, *ne1ne1Ne2Ne2*) × 'Yangbaimai' (YBM, *Ne1Ne1ne2ne2*). The two parents exhibited normal growth, and 24 RILs showed variable levels of hybrid necrosis. This RIL population was used for mapping *Ne1* and *Ne2*. Two lines (RIL-45 and RIL-128) with *Ne1ne1Ne2Ne2* genotype in this population showed segregation for normal and necrotic plants. Homozygous F_9 individuals (*ne1*^{YBM}*ne1*^{YBM}*Ne2Ne2* or *Ne1*^{ZN17}*Ne*^{ZN17}*Ne2Ne2*) from RIL-45 and RIL-128 were identified with markers and self-pollinated to produce near-isogenic lines (NIL-*Ne1*^{YBM} and NIL-*ne*^{ZN17}).

An additional RIL population (F_8) from cross 'Zhengzhou 6903' × 'Yumai 14' was developed for validating a 2.89 Mb deletion. A panel of 501 common wheat varieties (200 landraces and 301 cultivars, Supplementary Table S1) and 40 tetraploid wheat varieties (Supplementary Table S2) was used to study the frequency of *Ne1* in wheat germplasm. Five cultivars (carriers of known alleles of *Ne1* or with *Ne2*/*Lr13*) were used to check the accuracy of a newly selected marker (Supplementary Table S1).

Field experiments and phenotypic evaluation

The necrosis of the RIL population was evaluated in three replicates at two locations, Beijing (40° 16′ N, 116° 24′ E) and Zhaoxian county in Hebei province (37° 50′ N, 114° 49′ E), during two growing seasons (2016–17 and 2017–18). The populations used for fine mapping of *Ne1* were grown at the latter site in 2017–2018. Each line was represented as a single one-meter row plot with 11 plants, 25 cm between rows, and 10 cm between plants. Necrosis was scored at the grain fill stage.

Association analysis for *Ne* loci

Genomic DNA was extracted from young leaves by the CTAB method (Chatterjee et al. 2002). The parents and 188 RILs were genotyped with the wheat660K SNP array (https://wheat.pw.usda.gov/ggpages/topics/Wheat660_SNP_array_developed_by_CAAS.pdf). An association analysis between polymorphic SNPs and necrosis scores was conducted with the function of GLM (Generalized Linear Model) implemented in the software TASSEL Version 5.0 (https://tassel.bitbucket.io/). The threshold *P*-value was set to 3.42×10^{-7} to select associated SNPs based on the Bonferroni adjustment of independent SNPs. Genome coordinates presented in the study were based on the Chinese Spring RefSeq v1.0 assembly, and gene annotations were based on the updated RefSeq v1.1 annotation (IWGSC 2018).

Molecular marker development

For fine-mapping *Ne1*, SSR markers were designed from an ~ 290–400 Mb region on chromosome arm 5BL using BatchPrimer3 (http://batchprimer3.bioinformatics.ucdavis.edu/) based on the Chinese Spring RefSeq v1.0 assembly IWGSC 2018, and dCAPS markers were developed by dCAPS Finder 2.0 (http://helix.wustl.edu/dcaps/dcaps.html) based on the flanking sequence of the wheat660K SNP array. The 10 µl PCR system contained 2 µl DNA template (~ 40 ng/µl), 2 µl H_2O, 0.5 µl of 10 µM sense and 10 µM antisense primers, and 5 µl 2 × Taq PCR Starmix (GeneStar, China). PCR was done as follows: 5 min at 94 °C; 7 cycles of 30 s at 94 °C, 30 s at 63–56 °C (dropping 1 °C per cycle), 30 s at 72 °C; 25 cycles of 30 s at 94 °C, 30 s at 56 °C, 30 s at 72 °C; 3 min at 72 °C. PCR products were separated on 5% agarose or 12% non-denaturing PAGE gels. Primers used in the study are listed in Supplementary Table S3.

Results

Ne1 induced the necrotic phenotype and yield decrease in the presence of *Ne2*

The necrotic phenotype in NIL-*Ne1*^{YBM} first appeared on the lower leaves at the tillering stage (Fig. 1a) and became more evident at the heading stage in the field. More detailed observations indicated that leaf necrosis initiated from the bottom leaves and progressed gradually to the upper leaves, and advanced from the tip to base of the leaf (Fig. 1b). Necrosis formation in NIL-*Ne1*^{YBM} leaves appeared to follow a developmental pattern, in which the severity of necrosis was positively correlated with the age of leaves. At the grain fill stage, only the flag leaf or upper leaves remained partially green, and the rest of leaves were necrotic or dried in the NIL-*Ne1*^{YBM}, while at the same stage the leaves of NIL-*ne1*^{ZN17} remained green and healthy (Fig. 1c). In addition, the NIL-*Ne1*^{YBM} displayed smaller leaves (Fig. 1b) and fewer tillers, with a shorter plant height (Fig. 1c) compared to those of the NIL-*ne1*^{ZN17}. The premature senescence of leaves in NIL-*Ne1*^{YBM} resulted in significantly yield decline compared to NIL-*ne1*^{ZN17} (Fig. 1d).

Genetic mapping of Ne1

Necrosis was very evident at the grain fill stage, twenty-four of 188 RILs from ZN17 × YBM showed necrosis symptoms in all environments, whereas all the other 164 lines showed normal phenotypes. Therefore, association analysis was conducted using data from three replications grown in Zhaoxian county during 2016–2017.

The parents and 188 RILs of the ZN17 × YBM population were genotyped with the wheat660K SNP array. After removing the SNPs with > 5% data missing values and multiple mapping sites on RefSeq v1.0 (Keeble-Gagnere et al. 2018), 146,351 high-quality polymorphic SNPs were obtained. The associations of SNPs and the necrotic phenotype were made with the GLM function in TASSEL Version 5.0 software. There were 1,844 SNPs significantly associated with the phenotype at the threshold of *P*-value (3.42×10^{-7}). Among these SNPs, 916 (49.67%) and 870 (47.18%) were on chromosome arms 2BS and 5BL, respectively, and the remaining 58 (3.15%) were distributed across chromosomes 2A, 3A, 5A, 2D, 4D and 5D (Fig. 2a). These results were consistent with previous reports showing that hybrid necrosis in common wheat was controlled by *Ne1* on 5B and *Ne2* on 2B (Chu et al. 2006; Nishikawa et al. 1974; Zeven 1972). The significant SNPs on 5BL were in the ~ 297 Mb to 399 Mb region and those on 2BS located between ~ 110 Mb and 180 Mb (Fig. 2b).

Genetic analysis of segregating RILs and fine mapping of *Ne1*

Among a population of 252 individuals in RIL-45 (*Ne1ne1Ne2Ne2*), 53 plants showed severe necrosis, 134 had moderate necrosis, and 65 displayed a normal phenotype at the grain fill stage (Supplementary Fig. S1). This segregation fitted an expected 1:2:1 ratio ($\chi^2_{1:2:1} = 2.16$; $P < 0.05$).

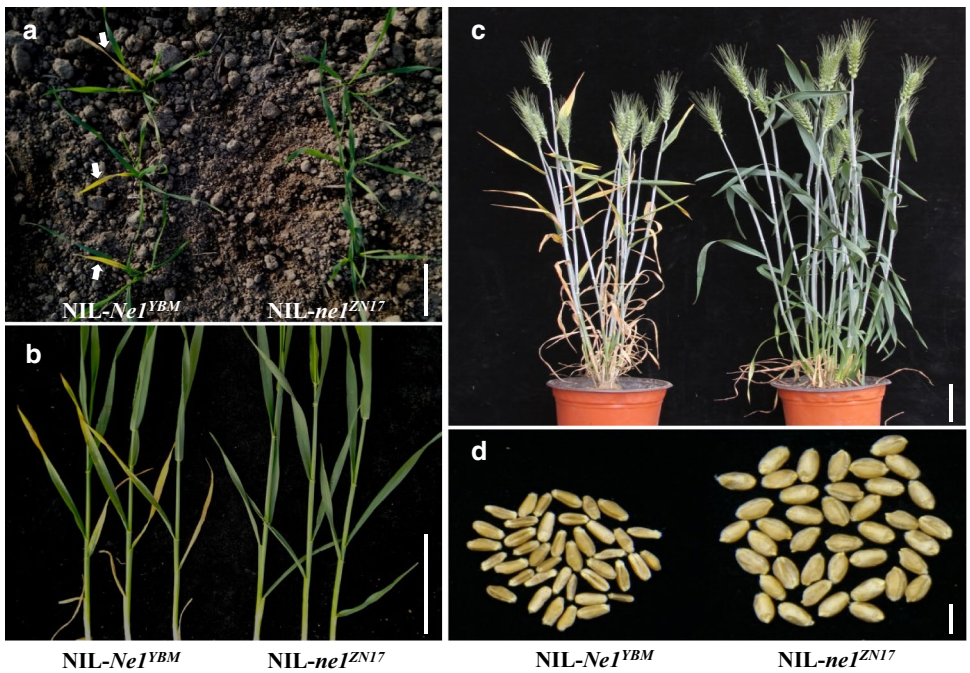

Fig. 1 Comparison of necrosis levels of individuals with different *Ne1* alleles derived from the segregating RIL-128. (**a**) The phenotypes of NIL-*Ne1*^{YBM} and NIL-*ne1*^{ZN17} at the tillering stage. (**b**) Morphology of the leaves at the jointing stage. (**c**) Phenotype of whole plant at the grain fill stage. Bar, 10 cm. (**d**) Seeds. Bar, 5 mm

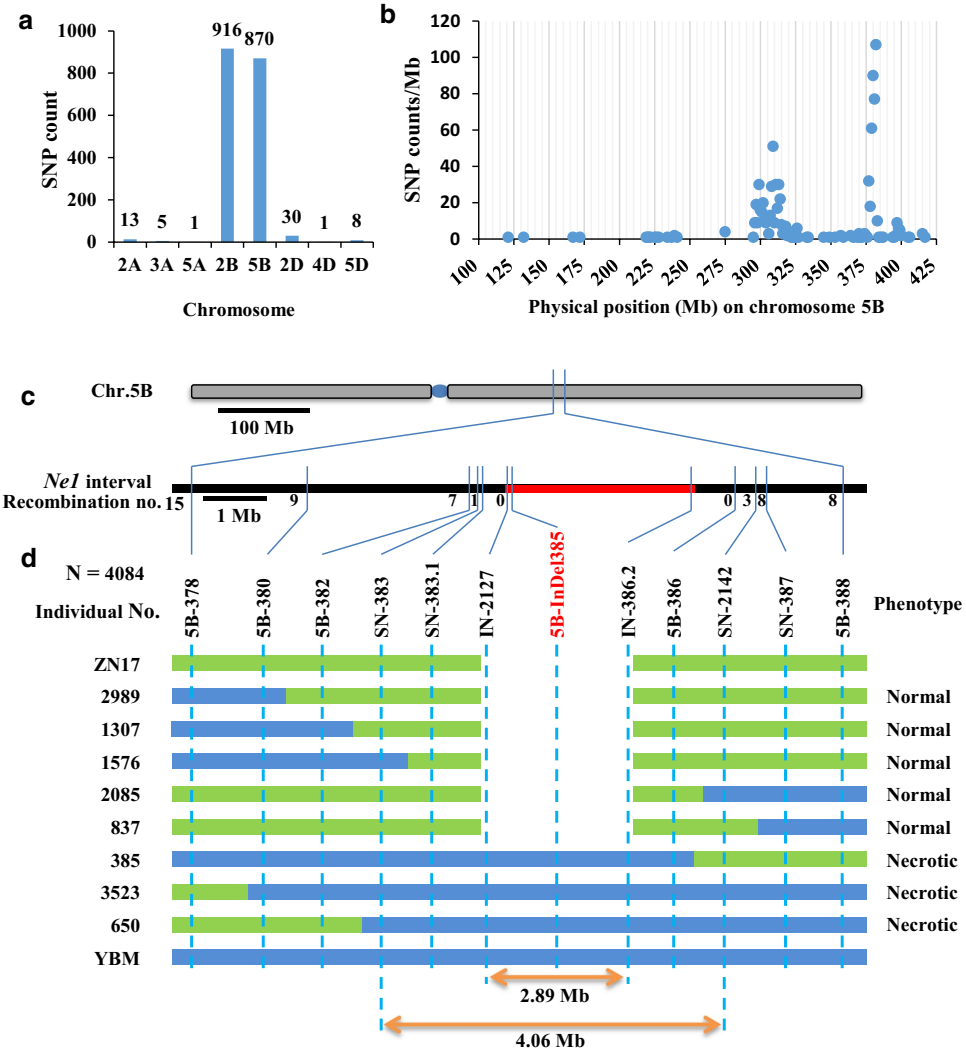

Fig. 2 Fine mapping of *Ne1* on chromosome 5B. Distribution of significantly associated SNPs in the whole genome (**a**), and on chromosome 5B (**b**) detected by GLM. (**c**) Physical distances between molecular markers; numbers in each region indicate the number of recombinants. Red block indicates the deletion in ZN17. (**d**) Graphical illustrations of the recombinant genotypes and phenotypes in the *Ne1* interval

These results indicated that the necrotic phenotype in this population was controlled by a semi-dominant gene.

Based on the IWGSC RefSeq v1.0 and flanking sequences of the associated SNPs, 50 molecular markers were developed for the *Ne1* region (~290–400 Mb) on chromosome arm 5BL; among them, four were polymorphic between ZN17 and YBM. These four markers were genotyped on 682 individuals in RIL-128. *Ne1* was delimited to a region flanked by markers *5B-378* and *5B-388* (Fig. 2c). A further 3,402 individuals from this population were analyzed to screen for additional recombinants; 23 recombinants were identified between the markers *5B-378* and *5B-388* (Fig. 2d). Based on re-sequencing data of both parents, we developed new polymorphic InDel and SNP markers and genotyped the recombinant individuals to narrow down the candidate interval of *Ne1*, which was eventually mapped to a 4.06 Mb physical interval delimited by markers *5B-383* and *SN-2142* (Fig. 2c, d).

The *ne1* allele in ZN17 was associated with a 2.89 Mb deletion on chromosome arm 5BL

In order to clone *Ne1*, we designed 57 SSR markers in the interval of ~383 Mb to 387 Mb between markers *5B-383* and *SN-2142* on 5BL based on IWGSC RefSeq v1.0. Most of the SSR marker variations between YBM and ZN17 were presence/absence. We speculated that this was caused by a deletion in ZN17. For confirmation, we re-sequenced ZN17 and YBM at 10X sequencing depths. Sequence reads were aligned to the reference genome of Chinese Spring (RefSeq v1.0), and SNPs and InDels located in the region between markers *5B-383* and *SN-2142* were called by the HaplotypeCaller module (Chai et al. 2018). We found a continuously missing sequence from ~383,441,497 to 386,325,646 (RefSeq v1.0) in ZN17, but not in YBM (Supplementary Table S4). This suggested that there was an approximate 2.89 Mb deletion between markers *5B-383* and *SN-2142* in ZN17. To verify the missing segment, we designed primers

to amplify six high confidence genes located in the putative missing segment of chromosome arm 5BL in the NILs and parents ZN17 and YBM (Supplementary Table S3). All six genes were detected in YBM and NIL-*Ne1*^{YBM}, but not in ZN17 and NIL-*ne1*^{ZN17}. These results further confirmed deletion of the *Ne1* candidate region in ZN17. Therefore, it was not possible to clone *Ne1* with the population developed from cross between ZN17 × YBM.

Development, validation and application of a selection marker for *Ne1*

To confirm that the missing 2.89 Mb fragment of chromosome arm 5BL was closely associated with *Ne1*, we screened a new segregating RIL (RIL-368, *Ne1ne1Ne2Ne2*) from the cross 'Zhengzhou 6903' × 'Yumai 14'. This population segregated for the necrotic phenotype in the field and showed the same PCR product pattern as RIL-128 with marker *5B-378*. We also identified all six genes located in the 2.89 Mb deletion in Yumai 14 (carrying *Ne1*), but not in Zhengzhou 6903 (carrying *ne1*). This indicated that deletion in *ne1* varieties might not be rare. We chose *5B-InDel385* as a predictive DNA marker for *Ne1* and divided wheat accessions into genotype *5B-InDel385_YBM* and *5B-InDel385_ZN17* based on the presence or absence, respectively, of the 2.89 Mb fragment.

To test the efficiency of the selection marker *5B-InDel385*, 1,034 individuals from RIL-368 were genotyped and analyzed. As expected, all plants with normal phenotype were *5B-InDel385_ZN17* and all necrotic individuals were *5B-InDel385_YBM*. These data confirmed that *Ne1* co-segregated with the marker *5B-InDel385* on chromosome arm 5BL.

The *5B-InDel385* was also validated in some carriers of known alleles of *Ne1* and *Ne2/Lr13*; including Kubanka (*T. durum*, *Ne1*^{s}), Chinese Spring (*Ne1*^{w}) and Sonalika, Manitou and Frontana (*Ne2*^{m}*/Lr13*). The marker *5B-InDel385* assay showed that the cultivars carrying *Ne2*^{m}*/Lr13* had the *5B-InDel385_ZN17* associated with the *ne1* allele. Kubanka and Chinese Spring, known to possess *Ne1* alleles, were *5B-InDel385_YBM* type. Based on these results, we conclude that the marker *5B-InDel385* could accurately distinguish *Ne1* from *ne1* which if included within the deletion would be a null allele.

Using the selection marker, we characterized 259 Chinese common wheat accessions (29 landraces and 230 cultivars). The *Ne1* allele was frequent in landraces (62%, Fig. 3a). Conversely, its frequency was much lower in modern cultivated wheat varieties (32%, Fig. 3b). We studied the geographical distribution of the *Ne1* allele in China and found that the frequency of wheat varieties containing the *Ne1* allele was highest in Henan (41.51%), followed by Shaanxi (41.38%), Shanxi (33.33%), Beijing (31.25%), Hebei (28.57%), Jiangsu (29.41%), and Shandong (5.13%; Table 1). Furthermore, 242 common wheat landraces/cultivars from diverse origin were detected with *5B-InDel385*. The results further confirmed that the 2.89 Mb deletion of the *Ne1* candidate region was widespread throughout the world. The *Ne1* allele was

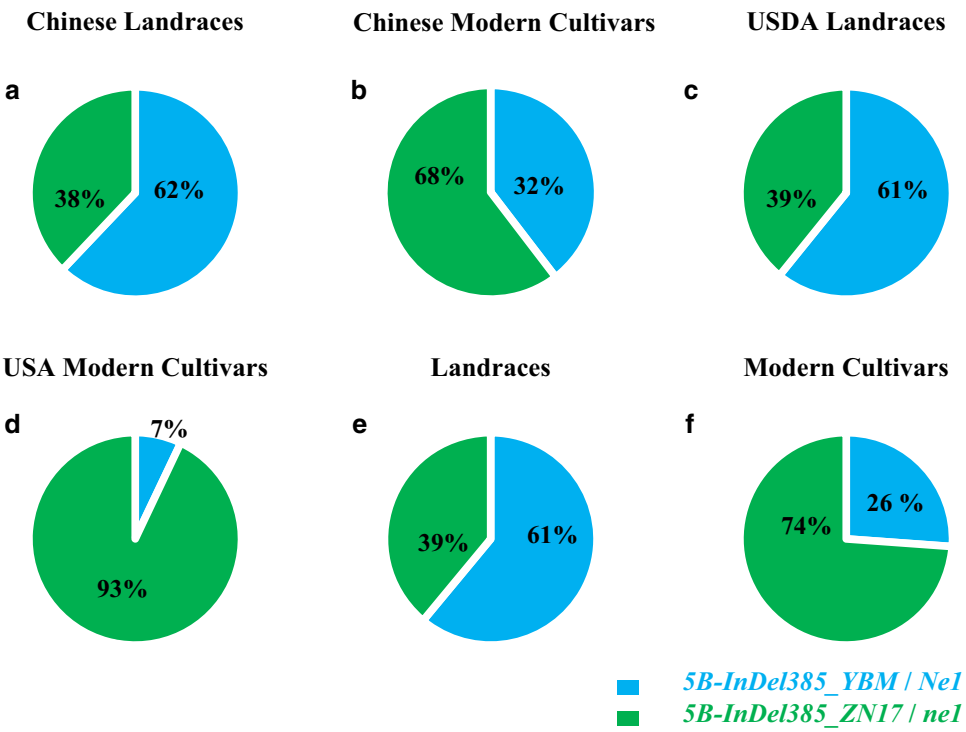

Fig. 3 Frequency of alleles *Ne1* (genotype *5B-InDel385_YBM*) and *ne1* (-2.89 Mb, *5B-InDel385_ZN17*) in wheat landraces and cultivars collected around the world. (**a**) and (**b**) Chinese landraces and modern cultivars. (**c**) South, West and Central Asian landraces obtained from the USDA collection. (**d**) Modern cultivars from the USA. (**e**) and (**f**) all landraces and cultivar accessions used to estimate allele frequencies

present in 104 of 171 landraces (61%) from USDA collections (from South, West and Central Asia; Fig. 3c). On the contrary, the *Ne1* allele was present only in 3 (7%) of a subset of 71 common wheat cultivars from the USA (Fig. 3d). In total, 122 landraces (61%) had *Ne1* (Fig. 3e) compared to 79 modern cultivars (26%) (Fig. 3f). This result suggested that the *Ne1* allele had been subjected to negative selection pressure in breeding. In addition, we also predicted the presence *Ne1* alleles in 40 tetraploid wheat varieties and found that 12 accessions were positive (Supplementary Table S2). All current results indicate that the deletion is frequent in both hexaploid and tetraploid wheat.

Putative *Ne1* candidate genes

We analyzed the predicted genes on the chromosome arm 5BL from 383.03 Mb to 387.10 Mb of the Chinese Spring RefSeq v.1.0 sequence to identify candidate gene for *Ne1*. Fifty-four genes were identified in the region; 28 were in the deleted 2.89 Mb region, 6 were in the region between marker *5B-383* and the deletion, and 20 were between marker *SN-2142* and the deletion (Supplementary Table S5). Re-sequencing analysis of ZN17 and YBM showed that the main difference between these parents in the *Ne1* candidate region was the 2.89 Mb deletion and no amino acid difference was identified in the 26 genes outside of the deletion bin although there were 385 SNPs/InDels present in the entire candidate interval (Supplementary Table S6).

Table 1 *5B-InDel385* genotypes of 230 wheat cultivars from China

Agro-ecological region	No. of accessions	*5B-InDel385*			
		5B-InDel385_YBM (*Ne1*)		*5B-InDel385_ZN17* (*ne1*)	
		No. of accessions	Percentage with *Ne1*	No. of accessions	Percentage with *ne1*
Henan	53	22	41.51%	31	58.49%
Shaanxi	29	12	41.38%	17	58.62%
Shanxi	15	5	33.33%	10	66.67%
Beijing	16	5	31.25%	11	68.75%
Hebei	42	12	28.57%	30	71.43%
Jiangsu	18	5	27.78%	13	72.22%
Shandong	39	2	5.13%	37	94.87%
Sichuan[a]	11	8	–	3	–
Anhui[a]	3	2	–	1	–
Zhejiang[a]	2	1	–	1	–
Yunnan[a]	2	0	–	2	–
Total	230	74	32.17%	156	67.83%

[a]These accessions were not included in determination of *Ne1* frequency due to limited numbers

To predict the *Ne1* gene, we analyzed the expression profiles of genes in the candidate region of Chinese Spring using the wheat expVIP expression platform (http://www.wheat-expression.com/). It was known that the necrotic phenotype was caused by interaction of the *Ne1* and *Ne2* alleles, and that 'Chinese Spring' carried the $Ne1^{w}$ allele (Hermsen 1963a; Zhang et al. 2016). Since symptoms of hybrid necrosis begin from an early seedling stage, we hypothesized that the *Ne1* allele should express in leaves and shoots for the entire growth period. Eighteen of 54 candidate genes expressed (above two transcripts per million) in at least three RNA-seq samples of Chinese Spring (leaves/shoots and roots, $n=40$) at different developmental stages (Supplementary Table S7). Nine of the 18 expressed genes were high-confidence genes, two encoding auxin-responsive SAUR proteins, two encoding serine protease HtrA-like proteins, two encoding alpha/beta-hydrolase superfamily proteins, one encoding an initiation factor 4F subunit (DUF1350), one encoding a RING/U-box superfamily protein, and one encoding a trypsin-like serine protease (Supplementary Table S8).

Discussion

The hybrid necrotic phenotype is influenced not only by gene dosage (dominance), but also by the genetic background

Wheat hybrid necrosis phenotypically manifests itself as premature gradual perishing of leaves, sheaths and even entire plants (Caldwell and Compton 1943; Hermsen 1963a). Hybrid necrosis is caused by the interaction of functional alleles at the complementary *Ne1* and *Ne2* loci (Hermsen 1963a), located on chromosomes 5B and 2B, respectively (Chu et al. 2006; Nishikawa et al. 1974). Previously, *Ne1* alleles were considered to be dominant based on the segregation ratio of necrotic: normal plants in F_2 populations derived from parents with genotypes *Ne1Ne1ne2ne2* and *ne1ne1Ne2Ne2* (Hermsen 1963a). *Ne1* had dosage effects (dominance) and *Ne1Ne1Ne2Ne2* genotypes developed more severe necrosis than *Ne1ne1Ne2Ne2* genotypes (Hermsen 1963b). In this study, we observed that heterozygous *Ne1ne1Ne2Ne2* individuals in segregating RILs were significantly different from homozygous *Ne1Ne1Ne2Ne2* sibs (Supplementary Fig. S1), confirming that *Ne1* was an incompletely dominant allele.

In addition, we found that the 24 of 188 RILs from ZN17 × YBM showed different degrees of necrosis indicating minor effects from other genes. Most of the necrotic lines exhibited necrosis at the leaf tips during the seeding stage; necrosis gradually progressed to the middle and bottom of the leaves at the jointing and flowering stages, and leaves

became totally necrotic at the late grain fill stage (Supplementary Fig. S2a-c). Three lines showed weak necrosis at the flag leaf tips at late jointing to flowering stage (Supplementary Fig. S2d). At the grain fill stage, six lines showed severe necrosis and shriveled grains caused by premature death of the leaves (Supplementary Fig. S2e); and two lines had yellow leaves (Supplementary Fig. S2f). This result implied that other genes were causing variation in symptoms other than *Ne*-gene interaction.

Allele *ne1* is likely to be null due to location of *Ne1* in a 2.89 Mb deletion

Microsatellite marker *Xbarc74-5B* was previously located 2.0 cM from *Ne1* (Chu et al. 2006). This marker is present in genome region 402,787,119 bp to 402,787,293 bp on chromosome 5B (Chinese Spring RefSeq v.1.0). In this study, we delimited the *Ne1* locus to a 4.06 Mb physical distance from 383.03 to 387.10 Mb (RefSeq v.1.0) and found that the *Ne1* allele segregated in repulsion with a 2.89 Mb deletion. Comparison of this region in multiple common wheat genomes revealed that this deletion was not rare (Supplementary Fig. S3) (Walkowiak et al. 2020). Based on the deletion we developed the selection marker *5B-InDel385*, which co-segregated with the *ne1* allele in large segregating populations (4084 plants). Because *5B-InDel385* is an InDel marker, it distinguished *Ne1* alleles from *ne1* among wheat germplasms, but not the different alleles of *Ne1*. Although Kubanka (*T. durum*, $Ne1^{s}$) and Chinese Spring ($Ne1^{w}$) carry different *Ne1* alleles (Zhang et al. 2016), both were classified as genotype *5B-InDel385_YBM*. Thus, we can select many germplasms with *ne1* using *5B-InDel385* to predict crosses that avoid hybrid necrosis.

We mapped *Ne1* to 383.03–387.10 Mb on chromosome arm 5BL (Chinese Spring RefSeq v.1.0). There were 54 annotated genes in this region, including 36 low confidence genes and 18 high confidence genes (IWGSC 2018). Resequencing of ZN17 and YBM revealed that 28 of the 54 genes were in a 2.89 Mb deletion in ZN17 and that another 385 SNPs/InDels caused no amino acid changes between the parents. Thus, it was likely that the expression differences of candidate genes led to the necrotic phenotype. We examined the expression profiles of all 54 genes in the 4.06 Mb genomic region of Chinese Spring and identified 9 high-confidence genes that were expressed in leaves/shoots or roots (Supplementary Tables S7, 8). We hypothesized that these 9 genes were candidates for *Ne1*. Gene annotation of the corresponding region in Chinese Spring revealed two auxin-responsive *SAUR* proteins and one initiation factor 4F subunit (*DUF1350*) (Supplementary Table S8). Rice plants overexpressing the *SAUR39* (one small auxin-up RNA) gene exhibited senescence of lower leaves beginning prior to initiation of the reproductive stage but extending upward with plant development (Surya et al. 2019). eIF4F is a complex formed by three proteins: eIF4A, eIF4E, and eIF4G and is tightly regulated to provide protection from abiotic and biotic stress. In Arabidopsis, double mutant eukaryotic translation initiation factors eIFiso4G1 (*i4g1*) and eIFiso4G2 (*i4g2*) exhibited pronounced premature senescence of primary rosette leaves long before completion of the life cycle (Lellis et al. 2010). However, based on the available data we could not determine the gene underlying *Ne1*. Therefore, quickest way to identify *Ne1* might be to individually knock out the nine most likely candidate genes in the presence of an *Ne2* allele. Disruption of the candidate gene should produce a normal phenotype. Although we associate the *ne1* phenotype with a large deletion that appears to be frequent, a search for an *ne1* haplotype that does not involve the deletion could enable map based cloning. An alternative procedure would be to identify a chemically induced mutant of *Ne1*. Once *Ne1* is cloned the basis of its allelic variation can be addressed.

The frequency of the 2.89 Mb deletion has increased during wheat breeding

Five hundred and one landraces/cultivars from diverse origin were analyzed with marker *5B-InDel385*. The frequency of *Ne1* carriers was higher in landraces, both from China and the USDA–ARS National Small Grains Collection (from Central, West and South Asia; Fig. 3a, c). In contrast, the frequency of *Ne1* was significantly lower (32%) in modern cultivated wheat varieties in China (Fig. 3b) and was only 7% in cultivars from the USA (Fig. 3d). This was consistent with Pukhalskiy et al. (2000), who reported that 9.1% of wheat cultivars in North and South-Central USA were *Ne1* carriers. Therefore, the frequency of *Ne1* was non-random due to negative selection pressure in breeding programs (Pukhalskiy et al. 2008, 2000). In addition, we found the 2.89 Mb deletion was also frequent in wild emmer wheat and durum wheat; an *Ne1* allele with the intact fragment was present in 12 (30%) of a panel of 40 tetraploid wheat accessions (Supplementary Table S2). This agreed with other researhers (Maccaferri et al. 2019; Zhu et al. 2019).

Since hybrid necrosis is the result of interaction between *Ne1* and *Ne2* alleles, the frequency of *Ne1* can be influenced by the presence of *Ne2* in wheat breeding programs. It is well known that the *Ne2* gene is tightly linked to the *Lr13* gene (a widely distributed leaf rust resistance gene in wheat) located on chromosome 2BS, and genetic and mutational analyses indicate that they are the same gene (McIntosh et al. 1995; Zhang et al. 2016). Thus, the fact that *Ne2* and *Lr13* represent a pleiotropic locus led to a sharp increase in the frequency of $Ne2^{m}$ in wheat in the USA, as *Lr13* was favored by selection for leaf rust resistance (Pukhalskiy et al. 2000; Zhang et al. 2016). The long-term use of $Lr13/Ne2^{m}$ may

explain the significant decrease in frequency of *Ne1* among USA cultivars that may have been selected to avoid hybrid necrosis.

Conclusion

Using large populations of selected RILs segregating at the *Ne1* locus and development of new molecular markers, the hybrid necrosis gene *Ne1* was mapped to a 4.06 Mb region (383.03 Mb–387.10 Mb) on chromosome arm 5BL and co-segregated with InDel marker *5B-InDel385*. Significant differences in *Ne1* frequency between landraces and modern cultivars predicted by the marker indicate that *Ne1* alleles have been subjected to strong negative selection in wheat breeding.

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Authors Contribution statement H-Q L and SZ conceived the project; SZ and YS developed the RIL and HIFs populations; YS carried out experiments and analyzed the data; ST and JN assisted in marker development and collected data for the RIL population; XS, SZ, YH and YS analyzed the data of re-sequencing; YL provided several cultivars-carriers of known alleles of gene *Ne1* or *Ne2*/*Lr13* and helped check the wheat accessions information; YS wrote the manuscript; H-Q L and SZ revised the manuscript.

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Availability of data and material All data generated or analyzed during this study are included in the main text article and its supplementary files.Code availability Not applicable.

Declarations

Conflicts of interest The authors declare that they have no conflicts of interest.

Ethics approval Not applicable.

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Consent for publication Not applicable.

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