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Molecular mapping of the hybrid necrosis gene *NetJingY176* in *Aegilops tauschii* using microsatellite markers

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ABSTRACT

The rich genetic variation preserved in collections of *Aegilops tauschii* can be readily exploited to improve common wheat using synthetic hexaploid wheat lines. However, hybrid necrosis, which is characterized by progressive death of leaves or plants, has been observed in certain interspecific crosses between tetraploid wheat and *Ae. tauschii*. The aim of this study was to construct a fine genetic map of a gene (temporarily named *NetJingY176*) conferring hybrid necrosis in *Ae. tauschii* accession Jing Y176. A triploid F_1 population derived from distant hybridization between *Ae. tauschii* and tetraploid wheat was used to map the gene with microsatellite markers. The newly developed markers *XsdauK539* and *XsdauK561* co-segregated with *NetJingY176* on chromosome arm 2DS. The tightly linked markers developed in this study were used to genotype 91 *Ae. tauschii* accessions. The marker genotype analysis suggested that 49.45% of the *Ae. tauschii* accessions carry *NetJingY176*. Interestingly, hybrid necrosis genotypes tended to appear more commonly in *Ae. tauschii* ssp. *tauschii* than in *Ae. tauschii* ssp. *strangulata*.

1. Introduction

Common wheat (*Triticum aestivum* L., 2*n* = 6*x* = 42, AABBDD) is a naturally formed allohexaploid species; tetraploid wheat (*Triticum turgidum* L., 2*n* = 4*x* = 28, AABB) provided the A and B genomes and the diploid goatgrass species *Aegilops tauschii* Coss. (2*n* = 2*x* = DD) provided the D genome approximately 8000 years ago [1,2]. As the source of the D genome of common wheat, *Ae. tauschii* shows abundant genetic variation useful for wheat improvement [3–9]. *Ae. tauschii* has historically been classified into two subspecies, ssp. *tauschii* and ssp. *strangulata* [3,10]. Elongated cylindrical spikelets are a prominent typical characteristic of *Ae. tauschii* ssp. *tauschii*, whereas quadrate spikelets are a prominent typical characteristic of *Ae. tauschii* ssp. *strangulata* [3,10]. The subspecies *tauschii* is distributed from eastern Turkey to China and Pakistan, whereas the subspecies *strangulata* is distributed only in the southeastern Caspian coastal region and Transcaucasia [3–5,33].

The rich natural variation in *Ae. tauschii* accessions, which remains largely unexplored, can be used for the improvement of wheat breeding through the production of synthetic hexaploid wheats [11–15]. However, abnormal phenotypes

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have been observed in many F_1 triploid hybrids between tetraploid wheat and *Ae. tauschii* [16–20]. Four abnormal phenotypes have been reported: type II and type III hybrid necrosis, hybrid chlorosis, and severe growth abortion [21]. Hybrid plants showing any of the four types of hybrid necrosis rarely produce selfed seeds [22]. Thus, in the process of transferring genes from related species to cultivated wheat, hybrid necrosis has been a serious obstacle [23,24].

Type II hybrid necrosis, which is characterized by tiller number increase and culm length decrease under low-temperature conditions, is one of the abnormal phenotypes observed in hybrids between tetraploid wheat and *Ae. tauschii* [22]. This type of necrosis is controlled by *Net1* and *Net2*, two complementary genes located on the A or B genome and the D genome, respectively [18]. *Net2* is located on the short arm of chromosome 2D, while the chromosomal location of *Net1* remains uncertain [14]. However, molecular markers closely linked to the two genes have not yet been identified. The tetraploid durum wheat (*T. turgidum* ssp. *durum*) cultivar Langdon has been extensively used as the AB genome source in creating synthetic hexaploid wheats [25] and carries *Net1* [26].

Simple sequence repeat (SSR) markers, also known as microsatellite markers, show highly polymorphic polymerase chain reaction (PCR) products in many plant species. In most cases, SSR markers are chromosome-specific and codominant. For this reason, they have been used in many areas of genetic analysis; for example, constructing genetic linkage maps [27–29], detecting genetic diversity [30], and mapping genes of interest [31,32]. Diverse breeding materials can be genotypically distinguished for phylogenetic studies by analysis of a small number of SSR markers [33,34]. A genome sequence of *Ae. tauschii* based on a whole-genome shotgun strategy has been published [35]. In addition, a physical map of *Ae. tauschii* covering 4 Gb has been developed [36]. The 1.72-Gb genome sequence and physical map of *Ae. tauschii* establish a foundation for developing SSR markers for fine mapping and map-based cloning of target genes in *Ae. tauschii*.

The aim of this study was to construct a fine genetic map of *NetJingY176* and to determine whether or not *Aegilops* accessions carry *NetJingY176*, using newly developed closely linked markers.

2. Materials and methods

2.1. Plant materials

Twelve *Ae. tauschii* accessions (CIae 9, CIae 17, CIae 25, PI 268210, PI 276985, PI 369527, PI 428564, PI 511375, Jing Y176, Jing Y199, Jing Y215, and Jing Y225) were used as pollen parents for crossing with the durum wheat cultivar Langdon (genome formula AABB). Tightly linked markers developed in this study were used for the identification of *NetJingY176* in 91 *Aegilops* accessions, including 49 *Ae. tauschii* ssp. *tauschii* and 42 *Ae. tauschii* ssp. *strangulata* accessions (Table S1).

To determine the chromosome location of *NetJingY176*, a triploid F_1 mapping population was developed. First an F_1 was obtained by crossing *Ae. tauschii* accession Jing Y225 (showing a normal wild-type (WT) phenotype when crossed with Langdon) with *Ae. tauschii* accession Jing Y176 (showing hybrid necrosis when crossed with Langdon). *Ae. tauschii* accessions Jing Y225 and Jing Y176 are classified as subspecies *strangulata* and *tauschii*, respectively (Fig. 1-a). Second, a mapping population with 199 triploid F_1 individuals was developed by crossing Langdon with the F_1 (Jing Y225/Jing Y176) using embryo rescue. The triploid F_1 carried the same AB genomes from Langdon as the female parent and a recombinant D genome derived from the hybrid of Jing Y176 and Jing Y225 as the male parents. After vernalization for 20 days at 4 °C, the triploid F_1 individuals to be used for phenotypic and genetic studies were planted in the greenhouse at Shandong Agricultural University, Tai'an, Shandong province, China.

2.2. Trypan blue staining

Each plant was grown at room temperature in the greenhouse for 3 weeks before staining. Leaves were harvested and placed in phosphate buffer solution (PBS), which was then mixed in equal volumes with 0.4% trypan blue dye solution. After incubation for 2 min in a boiling water bath, the leaves were cooled overnight. They were then bleached in 1.25 g mL^{-1} chloral hydrate for 3 d and examined under an inverted microscope [37]. Leaves stained with dye were considered dead.

2.3. DNA extraction and analysis of microsatellite markers

Genomic DNA was isolated from young leaves by the CTAB (Cetyltrimethyl Ammonium Bromide) method as described by Guo et al. [38].

Wheat microsatellite markers (*Xcfa*, *Xcfd*, *Xgdm*, *Xgwm*, *Xbarc*, *Xpsp*, and *Xwmc*) [27–29,38] on chromosomes 1D–7D based on previously published maps were chosen to analyze polymorphism between the two *Ae. tauschii* parents and the linkage relationships of *NetJingY176* in 10 plants showing normal phenotype (WT) and 10 plants showing hybrid necrosis using bulk segregant analysis (BSA). Wheat SSR markers on chromosome 2D were then selected for gene mapping. The sequences of the selected primers were obtained from GrainGenes 2.0 (http://www.wheat.pw.usda.gov/).

The PCR reaction mixtures of 15 μL contained 10 mmol L^{-1} Tris-HCl, pH 8.3, 50 mmol L^{-1} KCl, 1.5 mmol L^{-1} $MgCl_2$, 0.2 mmol L^{-1} dNTPs, 25 ng of each primer, 50–100 ng of genomic DNA, and 0.75 U of *Taq* DNA polymerase. DNA amplification was performed by denaturing template DNA at 94 °C for 5 min; followed by 35 cycles of 30 s at 94 °C, 30 s at 50–60 °C (depending on the annealing temperature of each primer), and 30 s at 72 °C, and 10 min at 72 °C as the final step. The PCR products were mixed with 6 μL of 6× loading buffer and separated by 8% nondenaturing polyacrylamide gels (39:1 acrylamide:bisacrylamide) by electrophoresis at 120 V for 3 h. Gels were visualized by silver staining as described by Guo et al. [38]. A genetic linkage map was constructed using JoinMap 4.0 [39] with a minimum LOD threshold of 2.0 for grouping order.

2.4. Development of new SSR markers closely linked to NetJingY176

Scaffolds of the *Ae. tauschii* 2D genome sequence [35,36] surrounding the necrosis gene *NetJingY176* were obtained

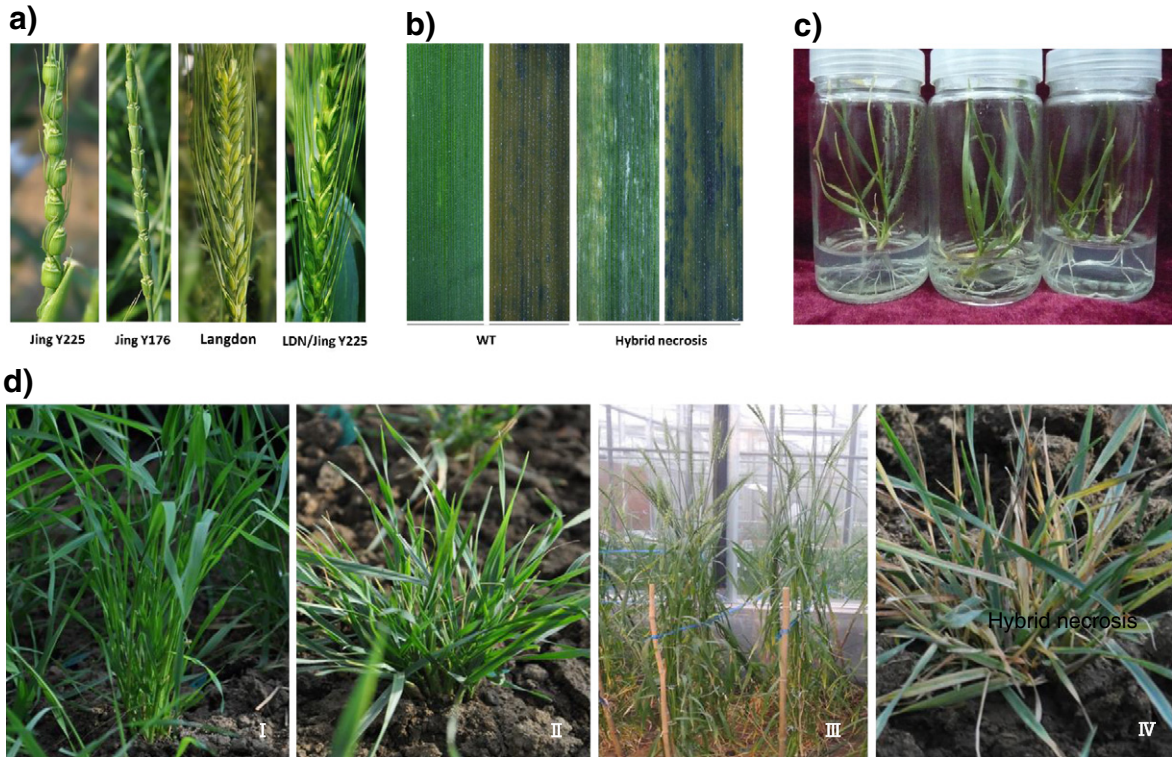

Fig. 1 – Phenotypic characterization of hybrid necrosis in an F_1 triploid individual. (a) Panicle traits of *Aegilops tauschii* accessions Jing Y225 and Jing Y176, tetraploid wheat cultivar Langdon, and a synthetic hexaploid wheat line (Langdon/Jing Y225). *Ae. tauschii* accessions Jing Y225 and Jing Y176 are classified as the subspecies *strangulata* and *tauschii*, respectively. (b) Phenotypic characterization of leaves by staining with trypan blue. Untreated leaves are on the left and leaves stained with trypan blue are on the right. (c) Triploid F_1 individuals in a constant-temperature incubator showed no obvious symptoms. (d) Phenotypic characterization of plants. I: Triploid F_1 individual with the wild-type phenotype at the seedling stage; II: Triploid F_1 individual with the hybrid necrosis phenotype at the seedling stage; III: Triploid F_1 individuals with the wild-type phenotype at the heading stage; IV: Triploid F_1 individuals with the hybrid necrosis phenotype stopped growing at the tillering stage and then died.

and used for marker development. The software SSR Finder (http://www.fresnostate.edu/ssrfinder/) was used to identify SSR regions in the scaffolds and sequences flanking the SSR region were selected to design SSR primers using Primer3 Input Version 0.4.0 (http://bioinfo.ut.ee/primer3-0.4.0/) under general settings. These primers were used to screen for polymorphisms between the two *Ae. tauschii* parents. Primer sequences of newly developed SSR markers are listed in Table S2. The primers were then used to screen for polymorphism between *Ae. tauschii* accessions Jing Y176 and Jing Y225. Marker analyses were performed as described above.

3. Results

3.1. *Phenotypic characterization of necrotic wheat—Ae. tauschii triploid hybrids showing necrosis*

Four of 12 F_1 hybrids from crosses between tetraploid wheat Langdon and four *Ae. tauschii* accessions (Jing Y176, PI 276985, PI 428564, and PI 511375) clearly showed an identical necrotic phenotype: dwarfing and markedly increased tiller number. The remaining hybrids showed normal growth.

Compared to WT, the phenotypic progression of hybrids with necrosis was divided into three stages based on plant height and leaf senescence (Fig. 1). In the first stage, no obvious necrotic symptoms were observed in a constant-temperature incubator at 4 °C for one month. In the second stage, after vernalization, triploid F_1 individuals were planted in the greenhouse at 24 °C/16 °C (day/night) for one month. Compared to WT individuals, hybrids with necrosis showed dwarfing and dramatically increased tiller numbers. Incomplete leaf expansion and stem tip necrosis were also observed at the tillering stage. In the third stage, the leaves of hybrids with necrosis gradually turned yellowish and the meristem gradually died, while the WT individuals produced selfed seeds.

The cell death response was observed in the leaves of the triploid hybrids. To further confirm this phenomenon, trypan blue was used to stain the necrotic leaves. No staining was observed in the leaves of WT individuals. In contrast, the leaves of hybrid necrotic plants showed extensive cell death (Fig. 1-b).

3.2. *Genetic analysis of the hybrid necrosis phenomenon*

A triploid F_1 mapping population was produced to map the gene for hybrid necrosis. In total, 199 triploid F_1 individuals

segregated as 110 WT and 89 hybrid necrosis plants, fitting a 1:1 segregation ratio (χ^2 = 1.114, P = 0.29). This result indicates that the gene controlling hybrid necrosis on the D genome is a single genetic locus. Thus, the phenotypic segregation of WT and hybrid necrosis in the triploid F_1 population can be used to confirm the genotypes of *Ae. tauschii* accessions Jing Y176 and Jing Y225 at the *NetJingY176* locus.

3.3. Marker development and molecular mapping of the gene *NetJingY176*

NetJingY176 on the D genome controlled hybrid necrosis in intraspecific crosses of tetraploid wheat and *Ae. tauschii*. To map the gene, 140 D-genome SSR markers were chosen to analyze polymorphism between the two *Ae. tauschii* parents and their linkage relationships of *NetJingY176* using BSA. Five markers on chromosome 2D were linked to *NetJingY176*. Then, 64 SSR markers that were previously mapped to chromosome 2D were investigated for polymorphism between *Ae. tauschii* accessions Jing Y225 and Jing Y176. Of these, only 16 identified polymorphic fragments were linked to *NetJingY176* in the population, suggesting that *NetJingY176* was located on the short arm of chromosome 2D. Markers *Xgwm102* and *Xgwm515* were located on each side of *NetJingY176* at genetic distances of 4.5 and 3.8 cM, respectively (Fig. 2).

Based on the genome sequence and the physical map of *Ae. tauschii*, 131 SSR markers were developed. Twenty-nine markers that were closely linked to *NetJingY176* were assigned to chromosome arm 2DS (Fig. 2) and 10 of them i.e., *XsdauK547*, *XsdauK554*, *XsdauK555*, *XsdauK558*, *XsdauK548*, *XsdauK534*, *XsdauK536*, *XsdauK541*, *XsdauK549*, and *XsdauK552* developed on the basis of *Ae. tauschii* scaffold sequences [35] were anchored on the chromosome 2D physical map constructed by Luo et al. [36]. Of these markers, *XsdauK539* and *XsdauK561* co-segregated with *NetJingY176* on chromosome 2DS and were designed from the same scaffold. The corresponding relationships between scaffold numbers and the new developed markers are presented in Table S2.

Comparative analysis was performed to assess the collinearity among *Ae. tauschii* 2D chromosome regions containing *NetJingY176* and *Brachypodium* (http://www.Brachypodium.org/), *Oryza* (http://rice.plantbiology.msu.edu/), and *Sorghum* (http://www.plantgdb.org/SbGDB/cgi-bin/blastGDB.pl). The results indicated that this syntenic region between *XsdauK552* and *XsdauK549* corresponds to *Brachypodium* chromosome 1 (*Bradi1g19627* and *Bradi1g19570*), *Oryza* chromosome 7 (*Os07g45160* and *Os07g45280*) and *Sorghum* chromosome 2 (*Sb02g041248* and *Sb02g041320*) (Fig. 2). Detailed comparative analyses revealed that only one gene, *Bradi1g19620* in *Brachypodium*, corresponding to *Os07g45170* in *Oryza* and *Sb02g041250* in *Sorghum*, is conserved. Thus, very lower levels of genomic collinearity were observed among *Brachypodium*, *Oryza*, and *Sorghum* in the *Ae. tauschii* chromosome region containing *NetJingY176*.

3.4. Identification of *Ae. tauschii* accessions putatively carrying *NetJingY176* using linked SSR markers

First, eight *Ae. tauschii* accessions showing the WT phenotype and four *Ae. tauschii* accessions showing hybrid necrosis phenotypes confirmed by hybridization were used to validate the efficacy of *NetJingY176*-linked markers *XsdauK536*, *XsdauK549*, *XsdauK555*, and *XsdauK560* for the identification of *Ae. tauschii* accessions putatively carrying *NetJingY176*. The marker assay indicated that the marker genotypes were in agreement with the phenotypes in all 12 *Ae. tauschii* accessions tested, so the markers closely linked to *NetJingY176* are useful for identifying the necrosis gene *NetJingY176* in *Ae.*

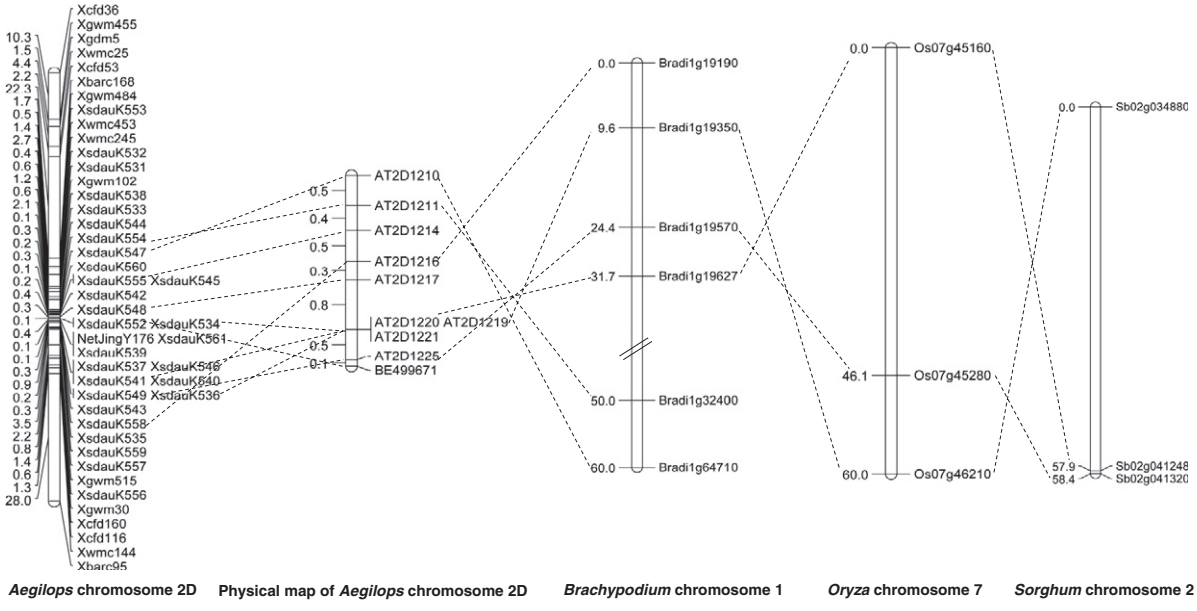

Fig. 2 – Comparative genetic linkage map of *NetJingY176* and its syntenic genomic regions on the chromosome 2D physical map, *Brachypodium* chromosome 1, rice chromosome 7, and sorghum chromosome 2, respectively. The units of these maps are cM, cM, 10 kb, kb, and 100 kb, respectively.

tauschii. Accordingly, the 91 *Ae. tauschii* accessions were screened with the linked SSR markers to detect the presence of *NetJingY176* gene, and the amplification bands for both WT and hybrid necrosis genotypes are shown in Fig. 3. Among the 91 *Ae. tauschii* accessions screened with markers *XsdauK536*, *XsdauK549*, *XsdauK555*, and *XsdauK560*, 45 showed hybrid necrosis marker genotypes and the remainder showed WT marker genotypes. The *Ae. tauschii* accessions with clear genotypes identified by SSR markers included 49 ssp. *tauschii* accessions and 42 ssp. *strangulata* accessions. Interestingly, of the 42 ssp. *strangulata* accessions, 30 showed WT marker genotypes and the rest showed necrosis marker genotypes. However, among the 49 ssp. *tauschii* accessions, only 16 showed WT marker genotypes and the other 33 showed hybrid necrosis marker genotypes (Fig. 4).

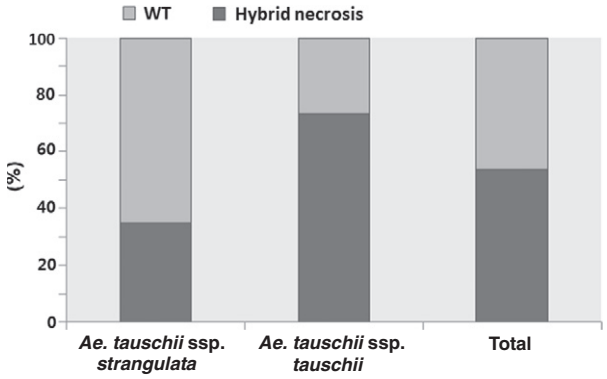

Fig. 4 – Distribution of the gene *NetJingY176* in *Ae. tauschii* ssp. *tauschii* and *Ae. tauschii* ssp. *strangulata*. The *Ae. tauschii* accessions comprised 49 ssp. *tauschii* and 42 ssp. *strangulata* accessions. Based on markers, 49.45% of the *Ae. tauschii* accessions showed hybrid necrosis genotypes, and 50.55% showed WT genotypes. Among 91 *Ae. tauschii* with clear genotypes, 28.57% of the ssp. *strangulata* accessions showed hybrid necrosis genotypes, whereas 67.35% of the ssp. *tauschii* accessions showed hybrid necrosis genotypes.

4. Discussion

Hybrid necrosis in wheat was first described in the 1940s [40]. Genes *Ne1* and *Ne2* are two dominant complementary genes that, when occurring together in a hybrid wheat, confer hybrid necrosis [41–44]. *Ne1* lies on the proximal half of chromosome arm 5BL, whereas *Ne2* lies on the distal half of 2BS [45]. The necrosis of wheat triploid F_1 hybrids was first mentioned by Nishikawa [16–18]. The tetraploid cultivar Langdon is an effective AB genome source for producing synthetic hexaploid wheat [12]. Large numbers of synthetic hexaploid wheat lines can be created with different D genomes from various *Ae. tauschii* accessions [46,47]. In synthetic wheat production, there are four types of hybrid abnormality [20,21]. Type II necrosis shows unique features, such as low temperature-induced necrotic symptoms and growth repression [22]. In this study, hybrid necrotic plants clearly exhibited a necrotic phenotype: dwarf plants and markedly increased tiller number after vernalization. This result suggests that the hybrid necrosis phenotype of triploid F_1 hybrids in the present study was that of type II necrosis.

Net2 is located on chromosome arm 2DS [14]. The molecular mapping performed in this study showed that gene *NetJingY176* from *Ae. tauschii* accession Jing Y176 co-segregated with the newly developed microsatellite markers *XsdauK539* and *XsdauK561* on chromosome arm 2DS. Comparison of the physical map of chromosome 2D [28] with the genetic map of 2D developed in this study shows that *NetJingY176* is physically located on the proximal half of the short arm. Two SSR markers *Xgwm102* and *Xgwm515* that flank *NetJingY176* are located in deletion bins 2DS-1-0.33-0.47 and 2DS-5-0.47-1.00, respectively. This result suggests that *NetJingY176* is likely to be identical to *Net2*. Thus, *Ae. tauschii* accession *JingY176* or other accessions carrying *Net* should not be used as parents when mapping populations for genetic research are created with synthetic hexaploid wheat lines. The closely linked microsatellite markers could be effective for identifying the genotypes of parental lines for *NetJingY176* or for using marker-assisted selection to remove the hybrid necrosis gene.

Gene deletion or insertion, which plays an important role in adaptation to stress and environment changes for plant species, is very common in the grass family [48,49]. In this study, comparative analysis was performed to investigate the collinearity among *Brachypodium*, *Oryza*, and *Sorghum* and the *Aegilops* chromosome 2D region containing *NetJingY176*. The results showed very low levels of genomic collinearity among these species in this *Aegilops* chromosome region containing *NetJingY176*. The low genomic collinearity could be explained by the better survival in unstable environments of species with gene deletions or insertions than of their ancestors [50,51].

Fig. 3 – Amplification bands of the wild type and hybrid necrotic plants with SSR markers. (a) Marker *XsdauK560*; (b) Marker *XsdauK549*. M: The size of an amplified fragment was estimated with a GeneRulerExpress DNA ladder; 1: Jing Y225; 2: Jing Y176; 3–12: amplification bands of the wild type (WT); 13–22: amplification bands of hybrid necrosis.

Determining the genetic and evolutionary relationships between the D genome of common wheat and the *Ae. tauschii* genome is crucial for understanding the development of common wheat. The *Ae. tauschii* genotypes associated with the origin of common wheat are confined to a narrow distribution range relative to the distribution range of the species, suggesting that *Ae. tauschii* harbors abundant genetic diversity that is not represented in common wheat [52]. In fact, only a few *Ae. tauschii* lineages were involved in the origin and development of common wheat. In addition, the wheat D genome is more closely related to that of *Ae. tauschii* ssp. *strangulata* than to that of *Ae. tauschii* ssp. *tauschii* [31]. In this study, 91 *Ae. tauschii* accessions were used to detect the *NetJingY176* gene. Based on the screening of SSR markers, 49.45% of the *Ae. tauschii* accessions showed necrosis marker genotypes and 50.55% of the *Ae. tauschii* accessions showed WT marker genotypes. However, among 91 *Ae. tauschii* accessions with genotypes revealed by the associated markers, 28.57% of the ssp. *strangulata* but 67.35% of the ssp. *tauschii* accessions showed necrosis marker genotypes (Fig. 4). Hybrid necrosis marker genotypes tended to appear more commonly in ssp. *tauschii* than in ssp. *strangulata*.

The molecular mechanisms of hybrid necrosis in synthetic hexaploid wheat are largely unknown. The mapping performed in this study has shown the location of the gene *NetJingY176* in the *Ae. tauschii* genome and opens the way for further elucidating the structure, products, and function of the hybrid necrosis genes. The distribution of *NetJingY176* locus in the various *Ae. tauschii* accessions may provide a guide for parental selection in use of *Ae. tauschii* germplasm collections for wheat germplasm enhancement and mapping population development.

5. Conclusion

We developed a triploid F_1 population by interspecific crosses between tetraploid wheat and *Ae. tauschii* to map the hybrid necrosis gene *NetJingY176*. The developed microsatellite markers *XsdauK539* and *XsdauK561* co-segregated with *NetJingY176* on chromosome arm 2DS. The tightly linked markers developed in this study are useful for identifying *Ae. tauschii* genotypes carrying *NetJingY176* and for cloning this gene from the *Ae. tauschii* genome. Genotyping analysis using SSR markers closely linked to *NetJingY176* revealed that 49.45% of 91 *Ae. tauschii* accessions potentially carry *NetJingY176*. Hybrid necrosis genotypes tended to occur more frequently in *Ae. tauschii* ssp. *tauschii* than in *Ae. tauschii* ssp. *strangulata*.

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Supplementary material

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