A Megabase-Scale Deletion is Associated with Phenotypic Variation of Multiple Traits in Maize

Xuesong Han, Yao Qin, Feng Yu, Xuemei Ren, Zuxin Zhang,¹ and Fazhan Qiu¹
National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan 430070, P.R. China
ORCID ID: 0000-0002-0709-9635 (F.Q.)

ABSTRACT Genomic deletions are pervasive in the maize (Zea mays L.) genome, and play important roles in phenotypic variation and adaptive evolution. However, little is known about the biological functions of these genomic deletions. Here, we report the biological function of a megabase-scale deletion, which we identified by position-based cloning of the multi-trait weakened (muw) mutant, which is inherited as a single recessive locus. MUW was mapped to a 5.16-Mb region on chromosome 2. The 5.16-Mb deletion in the muw mutant led to the loss of 48 genes and was responsible for a set of phenotypic abnormalities, including wilting leaves, poor yield performance, reduced plant height, increased stomatal density, and rapid water loss. While muw appears to have resulted from double-stranded break repair that was not dependent on intragenomic DNA homology, extensive duplication of maize genes may have mitigated its effects and facilitated its survival.

KEYWORDS Zea mays L.; megabase-scale deletion; pleiotropy; functional redundancy
two inbred lines. Among those genes, six genes are shared by B73 and McC, but four genes are lost in B73, showing loss of micro-collinearity at the DNA level (Fu and Dooner 2002). Genomic deletions also give rise to universal genetic effects. In rice (Oryza sativa), a 38.3-kb fragment harboring Grain number, plant height and heading date 
( Ghd7 ), which has a large effect on heading date and yield-related traits, is completely deleted in Zhenshan 97 but is present in Minghui 63 (Xue et al. 2008). A 254-kb genomic deletion reduces palmitic acid level in soybean (Glycine max L.) seeds (Goettel et al. 2016). Moreover, a 147-kb deletion containing maize wall-associated kinase (ZmWAK) in the major head smut quantitative resistance locus (named as qHSR1) confers susceptibility to the fungal disease head smut (Zuo et al. 2015). Additionally, genome-wide association studies (GWAS) show that SVs, including genomic deletions, are enriched at those loci associated with important traits, such as disease resistance, flowering time, plant height, and leaf-related traits in maize (Chia et al. 2012; Lu et al. 2015). These investigations provide vital links between genomic regions and phenotypes, and are shaping our understanding on the impact of genomic deletions as a major cause of plant phenotypic variation. Although massive genomic deletions have been identified by whole-genome sequencing in maize, little is known about the biological functions of these large-scale deletions.

To infer the potential mechanism of origin of genomic deletion, breakpoint junction sequences of massive genomic deletions have been detected from high-throughput sequencing data and been analyzed (Kidd et al. 2010; Jennes et al. 2011; Parks et al. 2015), and these investigations result in three hypotheses: nonallelic homologous recombination (NAHR), nonhomologous end joining (NHEJ), and microhomology-mediated replication-dependent recombination (MMRDR) (Chen et al. 2010; Jennes et al. 2011; Ottaviani et al. 2014). However, the molecular mechanism on the production of genomic deletions is still poorly understood in maize, except for the Ac/Ds transposable element system (Zhang and Peterson 1999). Moreover, homologous pairing of a large intercalary deletion and a normal complete homolog in heterozygotes, including wilting, decreased yield, reduced plant height, increased stomatal density, and accelerated water loss.

Materials and Methods

Plant materials

The maize spontaneous mutant muw with abnormalities in multiple characteristics was isolated from the maize Lian87 inbred line and crossed to another wild-type inbred line, V54, to develop the mapping population. To evaluate the effect of the muw mutation in different genetic backgrounds, the muw mutant was crossed to six maize inbred lines: B73, Mo17, 18599, Huangzaosi (HZS), HLZY, and Zong3. Among them, B73, HLZY, and Lian 87 are three inbred lines in Stiff Stalk (SS), Mo17 belongs to Non-Stiff Stalk (NSS), HZS belongs to Tang Si Ping Tou (TSPT), and Zong3 and 18599 are two inbred lines from mixed origins, respectively. The progenies of the six cross combinations were selfed separately to produce F2 individuals. To calculate genetic distance and recombination rate in the mapping region, two F2 populations, comprising 960 and 960 F2-individuals, respectively, were derived from the V54 × muw mutant and V54 × Lian87.

Water loss and chlorophyll efflux assay

To measure the rate of water loss, the second leaf from the top of the muw and wild-type plants (Lian87) at the seven-leaf seedling stage were collected and weighed, and then weighed once every 30 min for 6 hr at room temperature (26°C). The water-loss rate of the leaf was defined as the weight of water lost from the leaf at a specific time divided by the fresh weight (FW) with three replicates. Twelve leaves together were used for each measurement in three replicates.

The chlorophyll efflux assay was performed by measuring the permeability of the cuticle according to the description of Lolle et al. (1997) with minor modifications. Briefly, the second leaf from the top of muw and wild-type plants at the five-leaf seedling stage was collected and immersed in 96% ethanol in 50-ml tubes. These tubes were agitated on a shaker at 100 rpm. The chlorophyll in the supernatant was quantified using a UV 1800 spectrophotometer (Shimadzu, Japan) at wavelengths of 664 and 647 nm at six time points (0.5, 2, 4, 7, 12, and 24 hr) after initial immersion with three biological replicates. The chlorophyll efflux was expressed as a percentage of the chlorophyll amount at different time points to the total chlorophyll amount extracted after 24 hr of immersion.

Measurement of photosynthetic pigments

The measurement of photosynthetic pigments was performed according to the description of Arnon (1949) and Lichtenthaler (1987). Briefly, 200 mg FW of ear leaves at the filling stage were sliced and then collected into a 50-ml tube, followed by adding 25 ml of 95% ethanol, and the tubes were placed in the dark for 12 hr. The light absorption values of the extracts were measured at the wavelengths of 665, 649, and 470 nm by a UV 1800 spectrophotometer with three biological replicates, each replicate consisted of 12 plants from the muw mutant and wild type. Chlorophyll a (Chl.a, mg/g FW), chlorophyll b (Chl.b, mg/g FW), and carotenoid concentration (Car., mg/g FW) were then calculated.
phenotypic variation was performed using DNA Deletion Induces Phenotype Variation

Figure 1 Phenotypic comparison between the muw mutant and wild type. (A) Overall appearance of the wild type and the muw mutant plants after pollination. Bar, 20 cm. (B) Leaves from wild-type and muw plants after pollination. Bar, 10 cm. (C) Ears of the muw mutant and wild type. Bar, 2 cm. (D) Leaf water loss of the wild type and the muw mutant at different time points. Values are the mean ± SE, n = 3, 12 leaves per replicate. (E) Chlorophyll a (Chl.a, mg/g FW), chlorophyll b (Chl.b, mg/g FW), and carotenoid (Car., mg/g FW) concentration in wild-type and muw leaves at the seeding stage. PPC, photosynthetic pigment concentration. Data are shown as the mean ± SE, n = 12 individuals (*** P < 0.001, Student’s t-test). (F) Stomatal density in the lower epidermis of leaves of the wild type and the muw mutant under optical microscopy. Red and blue triangles indicate the stomata in the wild type and muw mutant, respectively. Stomatal density was detected in 20 individuals and is shown as the mean ± SE (*** P < 0.001, Student’s t-test). Bar, 100 μm.

by the equations published by Lichtenthaler and Wellburn (1983):

\[
\text{Chl.a (mg/gFW)} = 13.96 \times A665 - 6.88 \times A649 \\
\text{Chl.b (mg/gFW)} = 24.96 \times A649 - 7.32 \times A665 \\
\text{Car. (mg/gFW)} = (1000 \times A470 - 2.05 \times \text{Chl.a} - 114.8 \times \text{Chl.b})/245
\]

Abscisic acid (ABA) measurement and ABA response

Leaves (0.5 g) of muw and wild-type seedlings were collected, frozen in liquid nitrogen, and ground into fine powders, which were then collected into a centrifuge tube containing 4.5 ml of sample extraction buffer (Sun et al. 2017). The samples were lightly shaken overnight at 4°C and then centrifuged at 4°C for 10 min at 10,000 rpm. The supernatant was transferred into a new tube for measuring the concentration of ABA with an ABA ELISA kit (CUSABIO, College Park, MD).

To characterize the response of the muw mutant to ABA, we performed a germination assay as previously described with some modifications (Brugière et al. 2017). For muw mutant or Lian87 wild type, 100 seeds were soaked for 24 hr in sterile water (control) and 200 μM ABA (treatment) (Sigma, Santa Clara, CA), respectively, and soaked 24 hr in sterile water. Germinated seeds were scored after 2–5 days, and germination rates of seeds under the control and ABA treatment were counted with three replicates.

Cytological observations

Ear leaves were sampled from the muw and wild-type plants at the filling stage for measuring stomata number. The lower epidermis of the leaf was peeled off using tweezers and placed onto a microscope slide according to the description of Muir et al. (2014). Stomata were counted from five microscopic fields of the middle zone of the leaf. The stomata number per unit area of the muw and wild-type leaves was compared.

Stem internodes from the muw and wild-type plants were fixed in 4% paraformaldehyde (Sigma) overnight. The fixed tissue samples were dehydrated in a graded series of ethanol (30, 50, 70, 85, 95, and 100% ethanol), embedded in Paraplast Plus (Sigma), then sectioned into 8-μm slices using a Leica RM2265 microtome (Leica Microsystems, Wetzlar, Hesse-Darmstadt, Germany). The slices were stained using 0.5% toluidine blue and subsequently photographed using a Leica MZFLIII microscope (Leica Microsystems). The vascular bundles and vessels in the largest vascular bundle were counted from the photographed images.

Phenotyping agronomic traits

Four kernel traits, including 100-kernel weight (HKW), 20-kernel length (KL), 20-kernel width (KW), and 20-kernel thickness (KT), were measured using 60 muw mutant plants and 60 Lian87 wild-type plants with three repeated measurements. Four ear traits, including ear diameter (ED), cob diameter (CD), ear length (EL), and kernel row number (KRN), as well as plant height (PH) and ear height (EH) were also phenotyped in 60 muw mutant plants and 60 Lian87 wild-type plants.

Map-based cloning of the muw mutation and molecular marker development

To clone the muw mutation, we crossed the muw mutant to the V54 inbred line to create the mapping population. Primary mapping of the muw mutation was performed using
bulk segregant analysis (BSA). Thirty muw and 30 wild-type F2 individuals were separately pooled and then screened using ~1000 pairs of SSR markers that are chosen from the maize genome database (http://www.maizegdb.org/) and are distributed uniformly throughout the maize genome. For fine mapping, 14,496 F2 individuals were genotyped using markers flanking and locating in the mapping interval. Additionally, a total of 960 F2 individuals from each of two F2 populations (V54 × the muw mutant and V54 × Lian87) were genotyped with markers flanking the muw mutation to evaluate the recombination rate.

According to the BSA result, additional molecular markers (Supplemental Material, Table S1) were designed to narrow down the mapping region. The DNA sequences within the umc1485–umc1635 interval on chromosome 2 were retrieved from the B73 genome sequence (RefGen_V4) (www.maizegdb.org) and were searched for simple sequence repeats (SSRs) using the SSRHunter software (Li and Wan 2005). All sequences covering SSRs were then used for searching against the B73 reference genome; only those unique sequences were selected for future marker design. Meanwhile, flanking sequences of small insertion/deletion (InDel) mutations (3–7 bp) in the umc1485–umc1635 interval were downloaded from the Panzea website (www.panzea.org). Primer pairs were designed to generate PCR products of 100–200 bp using PrimerPlus version 4.0.0 (www.primer3plus.com) with the default settings. Mapmarker/exp3.0 software was used to reconstruct a linkage map (Lander et al. 1987).

Detection of deletion breakpoints and PCR product sequencing

Position of deletion breakpoints was estimated roughly using PCR amplification. If one primer pair can amplify a PCR product that we expected in wild type but cannot in the muw, we suggest that the DNA segment is deleted in the muw genome. Therefore, we can estimate roughly the deletion interval, and those markers mapped in the deletion interval, in this way. The left breakpoint was located in L6 and L7, and the right breakpoint was located in R11 and R12. Primers that flanked the detected deletion region, L6-F and R12-R, were used to conduct PCR on MUW and muw. A specific PCR product of ~12-kb that spanned the deletion suture point was amplified successfully in the muw mutant. The PCR product was then gel extracted and sequenced. Alignment of the PCR product sequence to the B73 V4 reference genome was performed to determine the exact breakpoint borders. The left and right breakpoints in the MUW were called at the edges of matching sequence. The deletion suture point in the muw was called where the broken ends are directly ligated.

The 12-kb amplicon was amplified by PCR from the genomic DNA of muw and sequenced on both strands with an ABI 3730 DNA analyzer (Tian Yi Hui Yuan Biotechnologies Co., Wuhan, China). For pairwise alignments and adenine and thymine contents analysis, two 2-kb segments near the left and right breakpoint were amplified by PCR from the genomic DNA of MUW and ABI sequenced. All PCR reactions for genomic DNA amplification were performed using Phanta Max Super-Fidelity DNA polymerase (Vazyme, Piscataway, NJ). The primers used are listed in Table S1. Sequence assembly and alignment was performed by CLC Sequence Viewer software (www.qiagenbioinformatics.com).

DNA extraction and genotyping

Genomic DNA of fresh leaves was extracted using the CTAB method (Saghai-Maroof et al. 1984). Genomic DNA of seeds was isolated using a previously described method with some modifications (Guan et al. 2012). Briefly, 0.02–0.05 g endosperms were cut from the apex of the seed, and were collected into the well of PCR plates. In each well, 50 μl 0.1 mol/L NaOH was added, followed by boiling for 10 min at 100°C in a thermal cycler. Then 50 μl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) buffer was added to each well, and the mixture was centrifuged at 2500 rpm for 1 min at room temperature. The supernatant was used as the template for PCR amplification. PCR products were subjected to electrophoresis on a 6% polyacrylamide gel, and then silver stained for visualization.

RNA extraction and quantitative reverse transcription PCR (qRT-PCR)

Leaves from four uniform seedlings that were planted in a plastic pot (5 cm in diameter and 6 cm deep) containing

| Table 1 Phenotypic comparison between the muw mutant and the wild type |
|-----------------------------|-----------------------------|-----------------------------|
| Trait                      | Wild type (WT) mean ± SDa   | Mutant (muw) mean ± SDa     | P-valueb |
| Kernel thickness (mm)      | 9.94 ± 0.27                 | 9.74 ± 0.21                 | 0.07     |
| Kernel length (mm)         | 21.32 ± 0.27                | 16.86 ± 0.33                | 1.45E–17 |
| Kernel width (mm)          | 19.04 ± 0.36                | 16.64 ± 0.25                | 1.73E–13 |
| 100-kernel weight (g)      | 29.52 ± 0.59                | 23.33 ± 0.41                | 1.37E–31 |
| Ear diameter (mm)          | 50.90 ± 1.59                | 43.68 ± 1.89                | 4.90E–06 |
| Cob diameter (mm)          | 29.55 ± 1.20                | 26.56 ± 1.40                | 1.41E–08 |
| Ear length (mm)            | 138.35 ± 2.33               | 116.05 ± 1.91               | 3.19E–29 |
| Kernel row number          | 16.2 ± 1.01                 | 16.1 ± 0.99                 | 0.77     |
| Plant height (cm)          | 165.05 ± 1.69               | 152.05 ± 2.91               | 3.12E–19 |
| Ear height (cm)            | 67.70 ± 1.10                | 62.50 ± 1.32                | 9.60E–16 |

a The mean values ± SD (SD, calculated from the variation observed over plants).

b P-values estimated by Student’s t-test. All traits were phenotyped in 60 individuals.
nutrient soil were pooled for RNA extraction using Trizol reagent (ThermoFisher Scientific, Waltham, MA), and the gDNA wiper Mix (Vazyme) was used to remove DNA. HiScript II qRT SuperMix II (Vazyme) was used to synthesize complementary DNA (cDNA). qRT-PCR was performed using the SYBR Green RT-PCR kit (ThermoFisher Scientific). The normalized expression levels were analyzed as described in Livak and Schmittgen (2001). Three biological replications were performed for muw and wild-type samples respectively. The gene-specific primers used are listed in Table S1.

Data availability

The authors state that all data necessary for confirming the conclusions presented in this article are represented fully within the article and Supplemental Material. Additionally, File S1 contains the 11,435-bp sequence flanking the deletion breakpoint (this sequence was also deposited with GenBank under accession No. MH675928). Plant materials in this study are available upon request. Supplemental material available at Figshare: https://doi.org/10.25386/genetics.7273028

Results

The muw mutant shows pleiotropic abnormalities in agronomic, cytological, and physiological traits

Phenotypic comparisons showed that the overall appearance of the muw mutant was highly similar to that of the wild-type individual (Figure 1A and Table 1), but the muw mutant was ~13 cm shorter than the wild type and exhibited dehydrated leaf tips beginning at the five-leaf seedling stage. The leaf tip dehydration phenotype was more serious with growth and development of the individual, resulting in irreversible damage to leaf tips and margins (Figure 1B). We also evaluated agronomically important traits carefully and found that the muw mutant produced a smaller ear relative to wild type. And EL, ED, and CD of the muw were significantly decreased relative to those of the wild type (by 16.1, 14.2, and 10.1%, respectively) (Figure 1C and Table 1). Moreover, compared to the wild type, KL, KW, and HKW of the muw were reduced by 20.9, 12.6, and 21.0%, respectively, which resulted in a smaller kernel size and a serious loss of grain yield in the muw (Table 1). However, the KRN and KT of the muw mutant did not differ significantly from that of the wild type (Table 1). Because excessive water loss usually leads to wilting in plants (Chen et al. 2004), we measured the water-loss rate and found 35.6–43.9% faster water loss in the muw leaves than in wild-type leaves (Figure 1D). Additionally, in comparison with the wild type, Chl. a and Chl. b concentrations in the muw leaves were 25.3 and 25.7% lower, respectively, but Car. concentration was 22.0% higher (Figure 1E), and the decreased Chl. a and b led to light-green leaves in the muw plants.

To understand the possible reasons for the increased water loss in the muw mutant, we characterized stomatal density, cuticle permeability, ABA concentration, ABA response, vascular bundle integrity, and water conductivity because these characteristics lead to wilting in plants (Sagi et al. 1999; Koizumi et al. 2007; Kim et al. 2014; Lee and Suh 2015; Y. Liu et al. 2015). Our results showed that the stomatal density of the muw leaves was 25.3 and 25.7% lower, respectively, but Car. concentration was 22.0% higher (Figure 1E), and the decreased Chl. a and b led to light-green leaves in the muw plants.

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S1A). In addition, differences in the ABA concentration between the muw mutant and the wild type were also not significant (Figure S1B), suggesting that the capacity to accumulate ABA under drought stress was not impaired in the muw mutant. Germination of the muw and wild-type seeds in response to exogenous ABA was also analyzed. Relative to control (seeds soaked with water), germination percentage of the muw seeds treated with exogenous ABA was decreased 10%, and germination of seeds was delayed. A similar phenomenon was also observed in wild-type seeds (Figure S1C). These results indicated that muw mutants exhibit a normal ABA response. Our analyses also showed that the number of vascular bundles (168.0 for the muw and 167.5 for WT) and vessels from the culm (2.18 for the muw and 2.16 for WT) were similar in both genotypes (Figure S1, D and E), which indicated that there were no significant differences in the ability to transport water between the muw mutant and the wild type.

Furthermore, to detect the effects of the muw mutation in different genetic backgrounds, we crossed the muw mutant to six maize inbred lines. All F1 plants of the six combinations exhibited wild-type phenotypes, and the F2 plants of each combination exhibited phenotypic segregation in agreement with a ratio of 3 wild type: 1 mutant producing wilted leaves (Figure 2 and Table S4), showing that the muw allele results in a similar effect in different genetic backgrounds, and is inherited as a single recessive locus.

A megabase-scale deletion is responsible for the multi-trait abnormalities in the muw mutant

To determine the breakpoints at both ends of MUW, we designed 17 markers (L1–L17, referred to as left-end markers) near M4, as well as 17 markers (R1–R17, referred to as right-end markers) near M5, and used these markers for screening the genetic polymorphism between the wild type and the muw mutant. If a pair of PCR primers can amplify the expected products in the wild type but not in the muw mutant, this indicates that the DNA segment may be absent in the muw mutant. Markers L1–L6 amplified the same sized products in the wild type and the muw mutant, whereas the other markers only amplified products in the wild type, indicating that the left breakpoint might be located between L6 (chr2: 70,464,146 bp) and L7 (chr2: 70,475,791 bp) (Figure 4A). Similarly, the right breakpoint might be located between R11 (chr2: 75,635,510 bp) and R12 (chr2: 75,636,014 bp) (Figure 4A). Therefore, we believe the deleted segment occurred between L6 and R12. To confirm these findings, we carried out a genomic PCR assay using primers L6-F and R12-R; a PCR product of ~12-kb was amplified in the muw mutant. However, the segment flanked by the L6 and R12 is too long to amplify product in the wild type (Figure 4B). After sequencing the PCR product, an 11,435-bp sequence was obtained and deposited in the NCBI database (File S1, Accession No. MH675928). A 10,757-bp long segment (1–10,757-bp from 5′- to 3′-end) of the 11,435-bp sequence was well matched with the sequence from 70,463,510 to 70,474,266-bp on chromosome 2 of B73 RefGen V4, and a 678-bp sequence (from 10,758 to 11,435-bp) was mapped to the segment from 75,635,981 to 75,636,658-bp on chromosome 2.

**Figure 3** Map-based cloning of the muw mutation. (A) The muw mutation was mapped to the umc1485 and umc1635 interval on chromosome 2 by bulked-segregant analysis. (B) The muw mutation was fine-mapped to a 5.66-Mb region flanked by M4 and M5. The numbers below and above the horizontal line, respectively, represent recombinational events and genetic distance (cM).
Sequence alignment revealed that a 5,161,714-bp (from 70,474,267 to 75,635,980-bp on chromosome 2 of B73 RefGen V4) sequence is entirely absent from the muw mutant’s genome. Thus, the left and right breakpoints for the deletion occurred between 70,474,266 and 70,474,267-bp and between 75,635,980 and 75,635,981-bp (Figure 4B) on chromosome 2 of the wild-type genome, respectively, based on the B73 RefGen V4. Within the deleted 5.16-Mb sequence, 48 genes are annotated in the B73 reference genome V4 (Table S2).

We then aligned the 11,435-bp sequence of wild-type allele muw immediately flanking the breakpoint against a reference collection of repeats using CENSOR software (Kohany et al. 2006), and found that, in the MUW allele, the flanking sequence of left breakpoint encodes a Ty1-copia-like maize retrotransposon, PREM-2 (pollen retroelement maize-2) (Turcich et al. 1996), which is bounded by two 1321-bp LTRs and contains coding domains of GAG (group-specific antigen), integrase (IN), reverse transcriptase (RT), and RNase H (RH) (Figure 4C). The LTR at the 3'-end of RH is absent in the muw allele. The left breakpoint in the MUW allele occurs between the 3'-end of RH and the neighboring LTR (Figure 4C). The flanking sequence of the right breakpoint harbors an incomplete gypsy-200 TE in the MUW allele, and the right breakpoint occurs in the internal portion of the gypsy-200 (Figure 4C). Thus, the muw mutant deleted the entire sequence between the PREM-2 and the incomplete gypsy-200 and parts of the PREM-2 and incomplete gypsy-200. Additionally, we retrieved a 1-kb DNA sequence on each side of each breakpoint from the MUW allele (File S2), and the 4-kb sequence (2-kb for each breakpoint) was used to conduct pairwise alignment and analysis of the percentage of adenine/thymine. The result showed only 41.3% sequence similarity, suggesting low sequence homology near the breakpoints. Moreover, the percentage of A/T is 69% in the upstream 200-bp region of the left breakpoint and 63% in the downstream 200-bp region of the right breakpoint, showing that the A/T content at breakpoint regions is more abundant than in regions 1-kb away from the breakpoints (Figure S2), and is also more abundant than the average A/T content (53.1%) of the maize genome (Jiao et al. 2017).

Transcripts for 20 genes in the deleted region are not detectable in the muw mutant

Expression data for these 48 genes retrieved from the qteller database (www.qteller.com) showed that all 48 genes are spatiotemporally expressed in different tissues and organs, or under abiotic conditions, indicating that they function in specific biological processes, cellular components, or stress responses. Among them, 20 genes that are expressed in leaf tissue were selected to measure their expression level by qRT-PCR. We found that these 20 genes were expressed in Lian87 leaves, but none was expressed in the muw mutant (Figure 4D). The gene expression results further exemplified the loss...
of these genes in the muw mutant. Furthermore, we attempted to amplify all 48 genes in Lian87, the muw mutant, and 60 other maize inbred lines using gene-specific primers. All of the 48 genes were amplified in Lian87 and in the 60 inbred lines, but not in the muw mutant (Table S3).

The abnormal phenotypes of the muw mutant may be associated with the loss of these genes. ZM00001d003980, which encodes a subtilisin-like serine proteinase, is an ortholog of Arabidopsis stomatal density and distribution1 (SDD1). Arabidopsis sdd1 mutants exhibit a twofold to fourfold increase of stomatal density and enhanced water loss (Berger and Altmann 2000; Vráblova et al. 2017). Overexpressing ZmSDD1 in maize decreases this water loss by reducing stomatal density (Y. Liu et al. 2015). Additionally, subtilisin-like serine protease-encoding genes are also specifically expressed in early developing kernels (López et al. 2017). Therefore, we suggest that ZM00001d003980 might be involved in stomatal and kernel development. ZM00001d003986 is a member of the leucine-rich repeat (LRR) receptor-like kinase superfamily. Some members of this superfamily, such as CLAVATA1 (CLV1), CLAVATA1-related BARELY ANY MERISTEM1 (BAM1), BAM2, and BAM3, are key regulators for cell proliferation and stem cell maintenance in dicots and monocots (DeYoung et al. 2006; DeYoung and Clark 2008; De Smet et al. 2009; Somssich et al. 2016). Another two deleted genes, ZM00001d003948 and ZM00001d003981, encode a putative DnaJ heat-shock protein and a DnaJ chaperone, respectively. DnaJ chaperones are involved in heat-stress tolerance in maize (Young et al. 2001; Sun et al. 2012; Klein et al. 2014; Hu et al. 2015). Despite a lack of direct evidence, we assume that the numerous phenotypic abnormities of the muw mutant are the combined effect of the loss of these genes encoded in the 5.16-Mb deleted region.

**Discussion**

In maize, plant height is highly related to internode development, which is determined by differentiation of the shoot apical meristem (Teng et al. 2013; Leiboff et al. 2015; Xing et al. 2015), and internode length, which is determined by the activity of the intercalary meristem (Hattori et al. 2009; Knöller et al. 2010). Plant stomata are derived from asymmetrical division of protodermal cells on the outermost tissue layer of the shoot apical meristem and symmetrical division of the guard mother cell (Bergmann et al. 2004). Kernel number and size are determined by inflorescence and floral development (Huang et al. 2009; Miura et al. 2010; Bai et al. 2016), cell division of the epispem during floral development, and the influx of carbohydrates after pollination (Song et al. 2007; Li et al. 2011; Zhang et al. 2012; Ishimaru et al. 2013). These biological processes are considered to be developmentally interrelated, and are controlled by different regulatory pathways. Here, we report a maize mutant with multiple abnormal phenotypes involving plant height, seed number and size, leaf stoma number, and other physiological characteristics, and is referred to as the **multi-trait weakened (muw)** mutant. Map-based cloning delimited the muw mutation into a 5.16-Mb deleted genomic segment, which is responsible for these defective phenotypes in the muw mutant.

**Deletion of a large DNA segment in the maize genome causes a wide range of phenotypic defects**

Genomic deletion is a universal genetic phenomenon. Cyto- genetic studies have shown that the deficiency in pale-yellow and white seedling mutants is associated with losses of terminal segments of the short arm of chromosome 9 (McClintock 1944). With the advance of next-generation sequencing, comparative genomics has revealed a massive amount of large-scale genomic deletions among maize genomes, which have played important roles in evolution and functional impacts (Saxena et al. 2014; Zhang et al. 2018). For example, the 147-kb deletion within qHSR1 from HZ4 contains ZmWAK, causing susceptibility to head smut in maize, and appears to have occurred after domestication and spread among maize germplasm (Zuo et al. 2015). Previous studies showed that loss of function of key genes leads to defective phenotypes involving multiple phytomers, and deletion eliminates gene function. In addition to deletion of key genes, several large deletions have altered a wide range of biological functions. The 1.2-Mb deletion induced by γ-irradiation within the opaque2 deletion line 107 resulted in loss of the 27- and 50-kD γ-zein-encoding genes and nine other genes on chromosome 7 (Yuan et al. 2014). In the mutant 1115, developed from B73 by γ-irradiation, a large deletion (~1.7 Mb) was identified on chromosome 3 by exome-seq and bulked segregant RNA-seq (BSR-seq), which covers 26 genes and leads to small and opaque kernels (Jia et al. 2016). In this study, the deletion of a 5.16-Mb segment on chromosome 2 in the muw mutant led to a loss of 48 genes, which was responsible for a set of phenotypic defects, including wilting leaves, poor yield performance, reduced plant height, rapid water loss, and increased stomatal density.

**Functional redundancy buffers the detrimental effects caused by deletion**

Some large-segment deletions, such as the 5.16-Mb deletion found in the muw mutant, do not cause severe defective phenotypes or lethality, which may be partially explained by the functional unimportance of a deleted gene and/or redundancy with other genes. First, megabase-scale presence/absence variations (PAVs) are frequently found when comparing the genomes of two diverse inbred lines. For example, 200 and 126 PAV sequences that were >5 kb were detected in B73 and Mo17, respectively. Among of these PAVs, a longest 2.9-Mb genomic segment harboring 66 annotated genes is present on chromosome 6 in B73 but is absent in Mo17 (Sun et al. 2018); however, Mo17 does not show any serious defective or lethal phenotypes relative to B73, despite the abundant quantitative variation between B73 and Mo17 in a wide range of phenotypic and physiological traits. Second, the functions of the missing genes can be fully or
partially compensated by their paralogs. A widely accepted view is that maize, as a paleopolyploid, underwent a recent whole-genome duplication followed by genomic diploidization involving complex chromosomal events (Bennetzen 2007; Wei et al. 2007). Although genes are rapidly silenced and/or lost from the duplicated genome, functional redundancy exists widely in the maize genome. For example, the duplicate SQUAMOSA promoter binding protein (SBP)-box transcription factors encoded by unbranched2 (ub2) and unbranched3 (ub3), function together redundantly to regulate KRN in maize (Chuck et al. 2014; Liu et al. 2015). Although the UB3-mum4 and UB2-mum3 single mutants do not show an obvious change in KRN, double mutants of UB3-mum4 and UB2-mum3 show a significant decrease in KRN relative to the wild type (Chuck et al. 2014). ALTERNATIVE DISCORDIA1 (ADD1) is 96% identical in amino acid sequence to DISCORDIA1 (Dcd1), which controls preprophase band formation in asymmetrically dividing cells in maize (Wright et al. 2009). Mutants of dcd1 have specifically misoriented asymmetric cell divisions in the leaf epidermis. Loss of function of add1 does not produce a noticeable phenotype, while knock down of add1 (RNAi) and dcd1 (RNAi) causes misorientation of symmetric and asymmetric cell divisions (Wright et al. 2009). Thus, the functional loss of a gene can be complemented by its closely related paralogs. In the B73 reference genome V4, the 48 genes lost in the muw mutant have a total of 460 paralogs, averaging 9.58 paralogs per gene. For example, a total of 45 putative subtilisin-like serine proteases are encoded in the B73 genome, and one, or several, of them might provide functional complementation to loss of ZM00001d003980 (Jiao et al. 2017). In addition, a total of 188 DnaJ proteins have been annotated in the B73 genome (Jiao et al. 2017). These putative paralogs may completely or partially complement the functions of the 48 deleted genes. Thus, functional redundancy of genes might partially explain the absence of severely detrimental or lethal phenotypes of the muw mutant.

Putative mechanism that caused the megabase-scale deletion

DNA double-strand breaks are the most harmful form of DNA damage. Large-scale deletions can occur during the repair of double-strand breaks by two main pathways: NAHR and NHEJ (Chen et al. 2010). The mechanism of genomic deletions may be associated with the DNA sequences at the downstream and upstream of the deletion breakpoints, which may help us to understand the mechanisms for creation of chromosomal deletions (Abeyesinghe et al. 2003; Gu et al. 2008; Jennes et al. 2011). NAHR is a type of genetic recombination that requires the presence of homologous DNA sequences (≥200 bp) to act as a template to directly repair the complementary strand (Jennes et al. 2011; Yang et al. 2016). For example, recombination between the internal regions of two barley retroelement (BARE) copies generates a recombinant BARE and a shortened copy with a 28-kb deletion in barley (Hordeum vulgare) (Shang et al. 2017); intrachromosomal recombination between two LTRs leads to a solo LTR in rice (Vitte and Panaud 2003). However, the lack of homologous or microhomologous sequences near the deletion breakpoints in the muw and the wild-type alleles makes it highly unlikely that homology-based mechanisms are involved. In other words, NAHR might not be a key mechanism causing the 5.16-Mb sequence deletion in the muw mutant. In contrast, NHEJ refers to the repair of double-strand breaks in which the broken ends are directly ligated (Yang et al. 2016). NHEJ is a major pathway for the repair of double-strand breaks in plant and mammalian cells (Gorbunova and Levy 1997; Lieber 2010), and very large deletions are thought to be generated by NHEJ (Sachs et al. 2000). In Arabidopsis, NHEJ repair of damaged DNA is the main mechanism involved in the formation of deletions (Britt 1996). Thus, NHEJ is likely responsible for the deletion in the muw mutant. Advances in the mechanism of genomic deletion depend mainly on research with human subjects (Chen et al. 2010; Jennes et al. 2011; Ottaviani et al. 2014). There are putatively three enzymatic activities required for repair of double-strand DNA breaks by the NHEJ pathway: (a) nuclease to remove damaged DNA, (b) polymerases to aid in the repair, and (c) a ligase to restore the phosphodiester backbone (Chen et al. 2010) (Figure S3). The mechanism of the 5.16-Mb deletion in the muw mutant still needs to be further studied. Additionally, adenine/thymine-enriched sequences are thought to play a role in the appearance of large deletions (Abeyesinghe et al. 2003; Jennes et al. 2011; Enggaard Hoeffding et al. 2014). The proportion of adenine/thymine in the flanking sequences of the breakpoint in the muw mutant is higher than the proportion of the whole genome, which may also be involved in the genomic deletion in this mutant.

Acknowledgments

This research was supported by the National Natural Science Foundation of China (91735306 and 31771796). We are grateful to Plant Editors for critically editing the manuscript. The authors declare no conflicts of interest.

Author contributions: F.Q. and Z.Z. conceived and designed the experiments; X.H., F.Y., X.R., and Y.Q. performed the experiments; X.H. analyzed the data; X.H., F.Q., and Z.Z wrote the manuscript.

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Communicating editor: A. Paterson