Inheritance of pre-emergent metribuzin tolerance and putative gene discovery through high-throughput SNP array in wheat (*Triticum aestivum* L.)

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**Abstract**

**Background:** Herbicide tolerance is an important trait that allows effective weed management in wheat crops in dryland farming. Genetic knowledge of metribuzin tolerance in wheat is needed to develop new cultivars for the industry. Here, we investigated gene effects for metribuzin tolerance in nine crosses of wheat by partitioning the means and variances of six basic generations from each cross into their genetic components to assess the gene action governing the inheritance of this trait. Metribuzin tolerance was measured by a visual senescence score 21 days after treatment. The wheat 90 K iSelect SNP genotyping assay was used to identify the distribution of alleles at SNP sites in tolerant and susceptible groups.

**Results:** The scaling and joint-scaling tests indicated that the inheritance of metribuzin tolerance in wheat was adequately described by the additive–dominance model, with additive gene action the most significant factor for tolerance. The potence ratio for all the crosses ranged between −1 and +1 for senescence under metribuzin-treated conditions indicating a semi-dominant gene action in the inheritance of metribuzin tolerance in wheat. The number of segregating genes governing metribuzin tolerance was estimated between 3 and 15. The consistent high heritability range (0.82 to 0.92) in F5–7 generations of Chuan Mai 25 (tolerant) × Ritchie (susceptible) cross indicated a significant contribution of additive genetic effects to metribuzin tolerance in wheat. Several genes related to photosynthesis (e.g. photosynthesis system II assembly factor YCF48), metabolic detoxification of xenobiotics and cell growth and development (cytochrome P450, glutathione S-transferase, glycosyltransferase, ATP-binding cassette transporters and glutathione peroxidase) were identified on different chromosomes (2A, 2D, 3B, 4A, 4B, 7A, 7B, 7D) governing metribuzin tolerance.

**Conclusions:** The simple additive–dominance gene effects for metribuzin tolerance will help breeders to select tolerant lines in early generations and the identified genes may guide the development of functional markers for metribuzin tolerance.

**Keywords:** Metribuzin, Gene effects, Inheritance, Potence ratio, Heritability, Wheat 90 K iSelect SNP genotyping assay, Candidate genes
Background

Wheat (Triticum aestivum L.) is a major global cereal crop in terms of production and area coverage (FAO 2018) [1]. Wheat is Australia’s largest grain crop and contributes around 12% of world trade. Western Australia (WA) has the highest reported occurrence of herbicide-resistant weeds in Australia, which is the key agronomic issue for WA farmers. There are instances where weed infestations have caused serious reductions (up to 50%) in wheat yields [2]. Higher tolerance for metribuzin is advantageous for WA wheat industry to protect crops against herbicide damage and maximize crop yields. Therefore, breeding wheat cultivars for higher herbicide tolerance through improvement programs is paramount, particularly in Mediterranean-type climatic regions.

Inheritance of metribuzin tolerance has a different mode of genetic control in crop plants. A monogenic recessive inheritance was reported in soyabean (Glycine max L.) [3, 4] and potato (Solanum tuberosum L.) [5]. Si et al. [6] reported two independent semi-dominant genes having additive effects in narrow-leafed lupin (Lupinus angustifolius L.). The inheritance of tolerance to metribuzin in durum wheat (T. turgidum L.) is a complex character controlled by both nuclear and cytoplasmic genes in wheat [7, 8]. This was supported by the observation that physiological processes, such as uptake, translocation and metabolism/detoxification, modified the amount of herbicide reaching the target site. Investigations into the genetic control and heritability of metribuzin tolerance will guide breeders to formulate the appropriate selection program for the breeding of herbicide tolerant cultivars.

Variation in metribuzin tolerance in wheat from six continents, reported in our previous investigation [9], provides a valuable source to breeders for estimating gene effects and formulating advantageous breeding procedures to improve herbicide tolerance. The natural variability observed between genotypes for metribuzin tolerance indicates that selection may be an effective method for improving yields. However, selection efficiency is related to the magnitude of heritability and genetic advances. Heritability estimates along with genetic advances are important selection parameters, and usually more helpful for predicting genetic gain under selection [10]. Therefore, a detailed understanding of the nature of gene action, heritability and predicted genetic gain is helpful for selecting superior wheat germplasm in breeding programs to improve herbicide tolerance and yield.

DNA markers have enormous potential for improving the efficiency and precision of conventional plant breeding via marker-assisted selection (MAS). The molecular mechanism of metribuzin tolerance in wheat is poorly understood. Advances in next-generation sequencing have facilitated the discovery of SNPs in the whole genome [11, 12] to provide a large amount of genome-wide polymorphism, as they potentially represent all the mutations that have occurred in the genome [13, 14]. The recent wheat 90 K SNP iSelect assay developed by Illumina is a useful genetic resource for tagging agriculturally important traits. The closed-end assay incorporates existing sequence knowledge onto a microarray platform enabling high-throughput SNP discovery in diverse pools.

This study aimed to (1) characterize the inheritance of tolerance to metribuzin in nine wheat crosses, (2) investigate heritability in F₅₋₋₇ RILs of the most diverse cross (Chuan Mai 25 × Ritchie) (3) conduct 90 K iSelect SNP genotyping assay in diverse cultivars to discover allelic variants in SNP markers in tolerant and susceptible groups, and (4) determine the likely chromosomal locations and candidate genes responsible for metribuzin tolerance in wheat.

Results

Phenotypic variation

The average senescence (SS) for the tolerant and susceptible parents used in this study are in Table 1. The susceptible parents had significantly (P < 0.05) higher SS than the tolerant parents. Average SS for F₁, F₂, BC₁T and BC₅ populations are in Table 2. The ANOVA indicated a highly significant difference between generations, indicating genetic variability for metribuzin tolerance in wheat. F₂ means had a comparable range to F₁ means. The mean SS of the backcrosses varied depending on the crossed parents. The abbreviations representing crosses are in Table 1. Backcrossing F₁ lines (BC₁T) to tolerant parents had lower SS than the mid-parent (mp) value, except for the K × D cross, indicating positive additive gene action and higher expression of metribuzin tolerance. In contrast, BC₅ had higher SS than the mid-parent value. The crosses of F₁ with susceptible Dagger differed the most from the mp value, 31.3, 38.6 and

| Cultivar | Origin | Senescence score | Reaction |
|---------|--------|-----------------|----------|
| Chuan Mai 25 (CM) | China, Asia | 3.05 ± 0.47 | T |
| Dagger (D) | Australia | 7.90 ± 0.25 | S |
| Eagle Rock (ER) | Australia | 3.95 ± 0.17 | MT |
| Fundulea 490 (F) | Romania, Europe | 4.40 ± 0.27 | MT |
| Kite (K) | Australia | 3.20 ± 0.34 | T |
| Ritchie (R) | Europe | 7.80 ± 0.32 | S |
| Spear (S) | Australia | 6.40 ± 0.11 | S |

*Pre-emergent metribuzin rate of 400 g ai ha⁻¹ was sprayed and phytotoxicity was measured in wheat seedlings, 21 DAT. See text for details about senescence scaling. Data represented are mean and standard error*
29% for the crosses CM × D, F × D and K × D, respectively. The comparisons of reciprocal crosses revealed significant differences (P ≤ 0.05) in average SS except for three reciprocal cross combinations (Table 3). Therefore the reciprocal crosses were not pooled for generation mean analysis.

### Table 2

| Cross (♀ × ♂) | Senescence score means | Potence ratio |
|--------------|------------------------|--------------|
|              | MP                     | F1           | F2           | BC<sub>T</sub> | BC<sub>S</sub> |              |
| CM × R       | 5.42                   | 3.64 (0.2)<sup>b</sup> | 4.45 (0.16)<sup>b</sup> (285)<sup>c</sup> | 3.35 (0.15)<sup>b</sup> (16)<sup>c</sup> | 6.77 (0.20)<sup>b</sup> (11)<sup>c</sup> | −0.75       |
| CM × S       | 4.72                   | 4.8 (0.2)<sup>b</sup> | 3.17 (0.11)<sup>b</sup> (343)<sup>c</sup> | 3.78 (0.11)<sup>b</sup> (16)<sup>c</sup> | 5.2 (0.11)<sup>b</sup> (10)<sup>c</sup> | 0.04        |
| CM × D       | 5.48                   | 5.86 (0.4)<sup>b</sup> | 5.4 (0.14)<sup>b</sup> (428)<sup>c</sup> | 5.0 (0.10)<sup>b</sup> (17)<sup>c</sup> | 7.20 (0.12)<sup>b</sup> (12)<sup>c</sup> | 0.16        |
| ER × R       | 5.87                   | 4.8 (0.37)<sup>b</sup> | 6.27 (0.09)<sup>b</sup> (692)<sup>c</sup> | 5.23 (0.15)<sup>b</sup> (13)<sup>c</sup> | 6.41 (0.13)<sup>b</sup> (12)<sup>c</sup> | −0.56       |
| ER × S       | 5.17                   | 5.8 (0.32)<sup>b</sup> | 6.24 (0.15)<sup>b</sup> (356)<sup>c</sup> | 4.09 (0.13)<sup>b</sup> (12)<sup>c</sup> | 5.68 (0.13)<sup>b</sup> (11)<sup>c</sup> | 0.51        |
| ER × D       | 5.92                   | 6.8 (0.42)<sup>b</sup> | 5.99 (0.12)<sup>b</sup> (412)<sup>c</sup> | 5.41 (0.13)<sup>b</sup> (12)<sup>c</sup> | 6.96 (0.17)<sup>b</sup> (12)<sup>c</sup> | 0.44        |
| F × R        | 6.10                   | 6.5 (0.37)<sup>b</sup> | 7.0 (0.16)<sup>b</sup> (428)<sup>c</sup> | 5.54 (0.16)<sup>b</sup> (15)<sup>c</sup> | 6.41 (0.13)<sup>b</sup> (12)<sup>c</sup> | 0.46        |
| F × S        | 6.10                   | 5.86 (0.79)<sup>b</sup> | 6.55 (0.12)<sup>b</sup> (476)<sup>c</sup> | 6.91 (0.19)<sup>b</sup> (17)<sup>c</sup> | 8.53 (0.15)<sup>b</sup> (13)<sup>c</sup> | 0.31        |
| F × D        | 6.15                   | 6.7 (0.25)<sup>b</sup> | 6.55 (0.12)<sup>b</sup> (476)<sup>c</sup> | 6.91 (0.19)<sup>b</sup> (17)<sup>c</sup> | 8.53 (0.15)<sup>b</sup> (13)<sup>c</sup> | 0.31        |
| K × S        | 4.80                   | 4.2 (0.58)<sup>b</sup> | 6.9 (0.09)<sup>b</sup> (356)<sup>c</sup> | 5.54 (0.16)<sup>b</sup> (15)<sup>c</sup> | 6.41 (0.13)<sup>b</sup> (12)<sup>c</sup> | −0.38       |
| K × D        | 5.55                   | 5.25 (0.41)<sup>b</sup> | 5.55 (0.11)<sup>b</sup> (475)<sup>c</sup> | 6.03 (0.18)<sup>b</sup> (13)<sup>c</sup> | 7.15 (0.15)<sup>b</sup> (10)<sup>c</sup> | −0.13       |

* MP = mid-parental value, calculated as (P 1 + P 2)/2, BC<sub>T</sub> and BC<sub>S</sub> represent backcross of F<sub>1</sub> to tolerant and susceptible parents, respectively
* Data not available
* Standard error
* Number of RILs
* Abbreviated cultivar names based on Table 1

** Genetic effects **

**Genetic model and gene action of metribuzin tolerance in wheat**

The results of the scaling tests (A, B, C and D) of nine hybrids (Table 4) were not significant, which indicated the absence of epistatic gene interaction and adequacy of the simple additive–dominance model. The genetic parameters for mp, additive gene effects (d) and dominance gene effects (h) and their standard deviations estimated by the joint–scaling test are presented in Table 4. The mp, which reflects the contribution of the locus effects and interaction of fixed loci, were significant for all nine crosses. The additive gene effects were significant (P = 0.05) for all nine crosses, and dominance gene effects were significant (P = 0.05) for four crosses (CM × R, CM × S, F × D and ER × D). The additive-dominance model fitted well for all crosses. The model significance was checked using χ² statistic, which showed insignificant difference between the expected and observed generation mean values, confirming a significant additive–dominance model for metribuzin tolerance in wheat (Table 4).

Metribuzin tolerance in wheat is either partially dominant or recessive dominant (Fig. 1). The potency ratio presented in Table 2 ranged from −0.75 to 0.51 for SS under metribuzin–treated conditions, thereby falling between −1 and +1, indicating a semi-dominant gene action for the inheritance of metribuzin tolerance in wheat. The crosses with a negative potency ratio (CM × R, F × S, ER × R, K × S and K × D) had lower F<sub>1</sub> means (lower phytotoxic effect) and were more similar to the tolerant parents, indicating the presence of partial
dominance gene effects. The crosses with a positive po-
tence ratio (CM × S, CM × D, F × R, F × D, ER × S, ER × 
D) had higher F1 means (higher phytotoxic effect), indi-
cating recessive dominance (Fig. 1).

Heritability and the number of resistance genes
The frequency distribution of the metribuzin reaction of 
F5–7 RILs of the Chuan Mai 25 × Ritchie appeared to be normal, indicating metribuzin tolerance as a quantitative 
trait (Fig. 2). The population means remained higher than those of the parents, indicating transgressive segregations in both directions of parents. Heritability was high and comparable in F5 (0.82), F6 (0.95) and F7 (0.92) RILs of the cross Chuan Mai 25/Ritchie (Table 5). There were minimum of eight major peaks representing major genes and some minor modifier genes in the F5, F6 and F7 RIL populations. Gene number, n1, estimated based on variances of parents and F2 and gene

| Cross (♀ × ♂) | Scales | Gene effects | \( \chi^2 \) |
|---------------|--------|--------------|--------|
|               | A      | B            | C      | D      | Mean (m) | Additive effect (d) | Dominance effect (h) | |
| CM × R        | 0.01 (2.54) | 2.10 (2.14) | −0.33 (10.92) | −1.22 (5.32) | 5.54 (0.20)** | 2.78 (0.18)** | −1.67 (0.36)** | 1.21 (NS) |
| CM × S        | −0.29 (2.34) | −0.80 (0.96) | −6.37 (8.96) | −2.64 (4.36) | 4.88 (0.15)** | 1.49 (0.13)** | −1.66 (0.34)** | 7.43 (NS) |
| CM × D        | 1.09 (2.48) | 0.64 (1.78) | −1.07 (10.18) | −1.40 (4.86) | 5.72 (0.19)** | 2.18 (0.13)** | 0.37 (0.38) (NS) | 0.58 (NS) |
| ER × R        | 1.71 (1.61) | 0.22 (1.90) | 3.73 (10.55) | 0.90 (5.19) | 5.92 (0.15)** | 1.65 (0.13)** | 0.09 (0.31) (NS) | 2.98 (NS) |
| ER × S        | −1.57 (1.60) | −0.84 (1.46) | 3.01 (11.80) | 2.71 (5.83) | 5.10 (0.09)** | 1.32 (0.08)** | 0.36 (0.22) (NS) | 1.81 (NS) |
| ER × D        | 0.07 (1.75) | −0.78 (2.19) | −1.49 (9.86) | −0.39 (4.70) | 5.82 (0.14)** | 1.80 (0.12)** | 0.64 (0.29)** | 0.00 (NS) |
| F × S         | 1.10 (2.58) | 0.82 (2.30) | 2.44 (12.56) | −2.18 (5.90) | 5.45 (0.12)** | 0.93 (0.10)** | 0.58 (0.27) (NS) | 1.64 (NS) |
| F × D         | 2.72 (2.13) | 2.46 (1.72) | 0.50 (11.32) | −2.34 (5.64) | 6.56 (0.15)** | 1.73 (0.13)** | 0.9 (0.29)* | 3.70 (NS) |
| K × D         | 3.61 (2.31) | 1.15 (1.86) | 0.60 (9.11) | −2.08 (4.38) | 5.88 (0.18)** | 1.86 (0.15)** | 0.14 (0.37) (NS) | 3.48 (NS) |

A, B, C, D, Scaling tests; \( \chi^2 \), Significance of the joint scaling test determined by the \( \chi^2 \) test and observed and expected \( 't' \) values compared at 5 and 1% level of significance.

** Indicates significant difference at \( P \leq 0.01 \); * Indicates significant difference at \( P \leq 0.01 \)

NS, not significant

\( \gamma \), Abbreviated cultivar names based on Table 1

Fig. 1 Dominance relationships between a pair of alleles A and B. Phenotypes corresponding to the different genotypes AA, AB and BB. -ve PR, negative potency ratio; F1 mean phenotypic value is similar to tolerant phenotypic value +ve PR, positive potency ratio; F1 mean phenotypic value is similar to susceptible phenotypic value.
number $n_2$, estimated based on variances of parents, $F_1$ and $F_2$ varied for most of the crosses. Wright’s formula estimated a minimum of three genes and a maximum of 15 genes controlling metribuzin tolerance in wheat (Table 6).

**SNP discovery and potential candidate genes**

The 90 K iSelect SNP genotyping assay contained 81,587 SNPs. A total of 60,635 monomorphic alleles (74%) with no clustering patterns for all genotypes were removed. A total of 12,294 loci had no call and were removed. The

| Population | No. lines | Range of SS | $\delta^2_g$ | $\delta^2_p$ | $\delta^2_e$ | $\delta^2_s$ | $H^2$ |
|------------|-----------|-------------|-------------|-------------|-------------|-------------|-------|
| $F_5$      | 73        | 1.7–10.0    | 32.29**     | 5.70        | 8.86        | 1.90        | 10.76  | 0.82  |
| $F_6$      | 73        | 2.0–10.0    | 110.44**    | 5.22        | 35.07       | 1.74        | 36.81  | 0.95  |
| $F_7$      | 73        | 1.7–10.0    | 60.73**     | 4.48        | 18.75       | 1.49        | 20.24  | 0.92  |

*Minimum and maximum senescence score

$\delta^2_g$, estimated genetic variance; $\delta^2_p$, estimated phenotypic variance; $\delta^2_e$, estimated error variance; $H^2$, broad sense heritability

$F_5$, $F_6$, $F_7$, single-seed descent recombinant-inbred lines of Chuan Mai 25 × Ritchie cross

** Indicates significant difference at $P < 0.01$
remaining 8,661 loci (12.9%) had ≥2 clusters and were used for principal component analysis (PCA) analysis; the results for allelic variation in seven genotypes are presented in Fig. 3. The PCA analysis revealed significant variation between tolerant and susceptible groups. A clear separation of tolerant and susceptible groups, according to PCA component 1, indicated high genetic diversity between the two groups. A total of 296 SNPs were polymorphic/biallelic markers between the two groups (Additional file 1: Table S1).

Putative genes related to the identified SNPs with differences between tolerant and susceptible groups were investigated by a blastN search of markers on *Triticum aestivum* IWGSC_refseqv1.0. The results suggested that metribuzin tolerance was a quantitative trait governed by several loci on different chromosomes (2A, 2D, 3B, 4A, 4B, 7A, 7B, 7D) (Table 7). Only genes related to photosynthesis and metabolic detoxification pathways were considered for candidate gene analysis. Multiple SNPs and candidate genes identified on chromosome 7B (photosynthesis system II assembly factor YCF48 and ABC transporter), chromosome 4A (cytochrome P450 family), chromosome 7A (glutathione S-transferase), chromosome 2A and 3B (glycosyltransferase), and chromosome 2D (glutathione peroxidase) represented the possible genes/gene families with significant association with metribuzin tolerance in wheat.

**Discussion**

The mode of inheritance and gene action of pre-emergent herbicide tolerance will help breeders to choose appropriate breeding methods to develop more tolerant cultivars and combat early weed competition to enhance wheat yields. The efficiency of selection and plant breeding programs depend on the existence of genetic variability [15]. Genetic variation for metribuzin tolerance in wheat was evident in our previous research [9, 16]. Metribuzin tolerance/sensitivity is controlled by both cytoplasmic and nuclear genes because reciprocal differences in expression of metribuzin tolerance existed in most F1 hybrids. Previously, Ratliff et al. [7] reported the role of both nuclear and cytoplasmic genes in metribuzin tolerance in wheat. Metribuzin tolerance is a polygenic trait and the present investigation revealed a maximum of 15 genes responsible for the trait. Villarroya et al. [8] reported metribuzin tolerance as a quantitative trait controlled by many genes in wheat, which supports the present findings. The Transgressive phenotypes observed in segregated populations (Fig. 2) compared to parental phenotypes were due to recombination of additive alleles both on positive and negative direction. Recombination results in new pairs of alleles at two or more

![Fig. 3](image-url) Principal component analysis showing genetic diversity based on 8,661 SNPs. Each point represents one individual. Principal component 1 (PC1) explains 28% of the variation and principal component 2 (PC2) explains 22.7% of the variation in the data
Metribuzin tolerance is explained by the simple additive–dominance model, indicating absence of epistasis or non-allelic interaction. The absence of epistasis and significant additive effect efficiently responds to selection [18]. The alleles of such traits are fixed in early generations. These facts can guide breeders in the selection of lines in early generations. The results of the scaling and joint-scaling tests and chi-square statistic can be used as evidence that the additive gene effect is higher than the dominance gene effect, indicating the former as a decisive type of gene action for metribuzin tolerance.

Highly significant additive gene effects (d) for all crosses indicated the preponderance of additive gene effects for metribuzin tolerance and the potential for improving the performance of chlorophyll traits using early a pedigree selection program in wheat.

### Table 7 List of 12 candidate genes with a known function related to photosynthesis and metabolic detoxification

| SNP name                     | Chromosome | A/B allele | Tolerant allele | Overlapping gene ID                      | Length (bp) and direction | Molecular function                                      | Biological process                                                                 |
|------------------------------|------------|------------|-----------------|-----------------------------------------|---------------------------|--------------------------------------------------------|-----------------------------------------------------------------------------------|
| wsnp_Ex_c13505_21253168      | 3B         | A/G        | B               | TRAES_3BF091600250CFD_c1                | 3614−                     | Glycosyltransferase activity                          | Sucrose synthase activity xenobiotics degradation                                 |
| Kukri_c5295_1015             | 3B         | T/G        | A               | TraesCS3B02G461800                     | 3614−                     | Glycosyltransferase activity                          | Sucrose metabolic process xenobiotics degradation                                 |
| BS00015680_51                | 2D         | T/C        | B               | TraesCS2D02G598000                     | 822−                      | Glutathione peroxidase (oxidoreductase, Peroxidase)    | Protection from oxidative damage                                                  |
| Kukri_c2937_649              | 2A         | A/G        | B               | TraesCS2A02G210100                    | 3125−                     | Glycosyltransferase activity                          | Metabolic detoxification/xenobiotics degradation                                   |
| CAP11_c3631_75               | 4B         | A/G        | B               | TraesCS4B02G056800                    | 1564+                     | Kinase and transferase activity                       | ATP-, metal-, magnesium- and nucleotide-binding                                    |
| BS00040929_51                | 7A         | A/G        | B               | TraesCS7A02G130600                    | 1495+                     | Glutathione S-transferase activity                    | Phase II metabolic isozymes involved in xenobiotic detoxification                 |
| Kukri_c1831_1243             | 4A         | A/G        | B               | TraesCS4A02G446700                    | 3084−                     | Sucrose synthase activity                             | Sucrose-cleaving enzyme that provides UDP-glucose and fructose for various metabolic pathways |
| tplb0060b03_921              | 7B         | T/C        | A               | TraesCS7B02G480500                    | 1549−                     | Photosynthesis system II assembly factor YCF48        | YCF48 is necessary for efficient assembly and repair of the PSII                   |
| RAC875_c16644_491            | 7D         | A/G        | A               | TraesCS7D02G268300                    | 1381+                     | Ubiquitination pathway                                | Stress response, DNA repair, signal transduction, cell-cycle control, transcriptional regulation and vesicular traffic |
| Tdurum_contig_10482_110      | 4A         | T/C        | A               | TraesCS4A02G445600                    | 1952+                     | Monoxygenase, oxidoreductase, iron and metal binding  | Cytochrome P450 family metabolize potentially toxic compounds including drugs and products of endogenous metabolism |
| GENE-1887_85                 | 3B         | T/G        | A               | TraesCS3B02G045400                    | 1437−                     | Oxidoreductase activity                               | Catalysis of oxidation-reduction reaction                                        |
| Tdurum_contig14460_561       | 7B         | T/C        | A               | TraesCS7B02G016400                    | 3807−                     | ATP- and nucleotide-binding; hydrolysis of ATP to energize diverse biological systems. | ABC module is known to bind and hydrolyze ATP in numerous biological processes including multiple drug resistance |

**Gene ID** is the TRAES number according to the URGI-Jbrowse database on Ensembl Plants release; +/− indicates the direction (forward/reverse) on the strand; bp indicates base pairs.
lower SS exhibited a partial dominant gene action. Therefore the F$_1$ hybrids with a negative potence ratio had mid- to low- metribuzin phytotoxic effects and expressed a phenotype similar to the tolerant parent (Fig. 1). However, F$_1$ hybrids with higher SS had recessive, dominant gene action. Therefore, the F$_1$ hybrids with a positive potence ratio had mid- to high- metribuzin phytotoxic effects and expressed a phenotype similar to the susceptible parent.

Heritability was consistent and above 80% in the F$_{5-7}$ RIL population of Chuan Mai 25 × Ritchie, which indicated stability of the metribuzin tolerance trait. These traits could be easily transferred through generations in breeding programs to generate more tolerant cultivar. The absence of epistasis increased the accuracy of the gene number estimate in the present study because it complied with Wright’s assumption of no epistasis [20]. The crosses had unidirectional distribution of genes based on the degree of susceptibility in susceptible parents. The crosses involving Ritchie as the susceptible parent segregated the most genes, followed by Dagger and Spear.

The candidate genes identified for SNPs having homoyzgous allele in the tolerant group encodes for the network of xenobiotic detoxification proteins protecting cells from oxidative damage and keeping the photosynthesis process intact by PSII complex repair under stress. The identified gene superfamilies or domains, notably cytochrome P450 (CYPs) and glutathione S-transferase (GSTs) glycosyltransferase (GT), ATP-binding cassette transporters and glutathione peroxidase (GPX) are essentially xenobiotic detoxifying enzymes involved in vacuolar sequestration of conjugated pesticide metabolites [21–23]. Plants can metabolize a diverse range of xenobiotics, such as organic pollutants and herbicides using enzymes [22]. The most commonly observed route for the detoxification of herbicides in wheat involves an initial hydroxylation, typically mediated by a cytochrome P450 mixed function oxidases (CYPs) and glutathione conjugation mediated by glutathione S-transferases (GSTs). CYPs and GSTs are implicated in metabolism-based resistance to multiple herbicides in grass weeds such as black-grass [24].

The identified glycosyltransferase and oxireductase mediate different biological processes. They are involved in sucrose metabolism and metabolic detoxification of xenobiotic detoxification. The candidate genes detected from our previous investigation [16] of QTL mapping suggested glycosyltransferase and oxidoreductase involved in metabolic detoxification, partially imparts metribuzin tolerance in wheat. The microarray analysis conducted by Pilcher et al. [25] revealed that sucrose metabolism was highly responsive to metribuzin stress in wheat. The identified photosystem (PS) II assembly factor YCF48 is the thylakoid-embedded large pigment-protein complexes of photosynthetic electron transfer chain, i.e. PSII, PSI, the cytochrome b$_6$f complex, and the ATP synthase. These multiport complexes harness solar energy and, together with ATP synthase, produce reducing power (NADPH) and chemical energy (ATP) for the production of carbohydrates in the Calvin cycle [26–29]. The ubiquitination pathway is involved in nitrogen recycling and prevents senescence during herbicide stress [30]. In conclusion, the proteins encoded by the identified genes are involved in the metabolic detoxification, carbon metabolism, and repair of the PSII complex.

Understanding the genetics of herbicide tolerance in wheat will guide breeders in the development of herbicide-tolerant cultivars with wider safety margins. Metribuzin tolerance in wheat has high heritability and significant additive gene action with no epistasis. Therefore, MAS may be a feasible routine solution for selecting herbicide-tolerant lines in crop improvement programs. Metribuzin tolerance in wheat is most likely a non-target-based mechanism where metribuzin is detoxicated by a series of metabolic enzymes. However, transcriptome-wide gene expression profiling is needed to reveal genes and pathways endowing metabolic herbicide resistance in wheat.

**Conclusions**

The simple additive-dominance mode of gene action suggests that a simple selection procedure could be successfully exploited in an early segregating generation to select lines for metribuzin tolerance breeding in wheat. The present investigation emphasized the degree of gene expression in the PSII assembly factor, antioxidants and detoxifying systems (CYPs, GSTs, GT, GPX) as the responsible factors for determining metribuzin tolerance in wheat. The identified markers could be used in marker-assisted selection of lines for breeding tolerant cultivars. Alternatively, tolerant genes could be introduced into elite wheat cultivars by natural introgression to enhance metribuzin tolerance.

**Methods**

**Herbicide**

Metribuzin (C$_8$H$_{14}$N$_4$O$_2$S), a triazinone herbicide was purchased from Syngenta Crop Protection. Metribuzin binds its target site D1 protein in PSII and inhibits electron flow between the primary electron acceptor to plastoquinone, arresting photosynthesis. The metribuzin dose of 400 g a.i. ha$^{-1}$ was used to assess tolerance status in parents, F$_1$, F$_2$, BC$_T$ and BC$_S$ populations and F$_{5-7}$ RILs of the cross, CM × R (for all abbreviations refer to Table 1).

**Plant material**

Seven wheat genotypes with differential tolerance to metribuzin (Table 1) were obtained from Australian
winter wheat collection. The tolerant and susceptible parents selected for this study were from previous tolerance screening [9] and local WA cultivars identified by Kleemann and Gill [31]. Plants of metribuzin T (tolerant) and S (susceptible) parental type were grown in 1 L pots containing potting mix (50% peat moss: 50% river sand) and maintained in a glasshouse at The University of Western Australia during a normal winter growing season. Single T and S plants growing individually in pots were paired according to floral synchronicity to produce F₁ maternal R and paternal S (F₁ RS) and F₁ maternal S and paternal R (F₁ SR) hybrids. Reciprocal crosses were used to check maternal effects of herbicide resistance. Subsequently, RS F₁s were selfed and backcrossed to their R and S plants to produce F₂ and backcross (BC₁ and BC₂) generations, respectively. Additionally, the Chuan Mai 25 (T) × Ritchie (S) cross was selected to develop recombinant inbred lines (RILs) in the growth chamber using rapid generation single seed-descent in-vitro embryo culture technique (Fig. 4) [32]. A total of 73 F₅–7 RILs were screened for metribuzin tolerance in the glasshouse to calculate heritability.

Herbicide screening and phytotoxic assessment
The parents, F₁, F₂, BC₁ and BC₂ populations and F₅–7 RILs of the cross, CM × R were evaluated for metribuzin tolerance in a sand-tray system [9]. The trays were sprayed with 400 g a.i. ha⁻¹ of metribuzin via a twin flat-fan nozzle, perpendicular to the tray surface in two passes at a flow rate of 118 L ha⁻¹ and 200 kPa pressure in a cabinet spray chamber. The amount of herbicide required for 400 g a.i. ha⁻¹ in L/ha was calculated using the ratio of herbicide rate by flowrate of twin flat-fan nozzle. The trays were maintained in a phytotron, where the temperature was set to 25/15 °C day/night and watered regularly every 48 h.

Senescence score (SS)/visual damage was measured 21 days after treatment (DAT) (Fig. 5). Plants with no visual symptoms were scored as 0, increasing levels of yellowing and stunting were scored from 1 to 4, increasing levels of leaf abnormalities (leaves wrinkling) and leaf necrosis were scored from 5 to 8, and dead plants with total leaf browning and necrosis of the apex were scored as 9. Lines with an average SS ≤ 3 recorded tolerant (T), 4 to 5 moderately tolerant (MT), and 6 to 9 as susceptible (S). For parents and F₁ hybrids, SS was averaged over the three repeats.

Identification of SNP and potential candidate genes
The distribution of alleles at the SNP sites was assessed using the wheat 90 K iSelect SNP genotyping assay, containing 81,587 genome-wide distributed SNPs following the procedure described by Wang et al. [33]. Allele calls were generated for the seven parents used in this study (Table 1), with the four tolerant genotypes as group 1 and three susceptible genotypes as group 2 for comparison. SNP clustering and genotype calling were performed using Genome Studio 2.0 software (Illumina). The monomorphic and
poor-quality SNP markers, which had more than 20% missing values, ambiguous SNP calling, or minor allele frequencies below 5%, were excluded from further analyses. The polymorphic SNP loci between the two groups were used for candidate gene analysis.

The candidate genes controlling metribuzin tolerance were identified using BLASTN program, against the Ensemble Plants (Triticum aestivum IWGSC_refseqv1.0) to find the Traes numbers of genes. BLAST hits were filtered with an e-value threshold of $10^{-5}$ and sequence similarity higher than 95%. The Traes numbers were searched in UniProt in TrEMBL (http://www.uniprot.org) and UniParc (https://www.uniprot.org/uniparc/) to obtain more information including protein domain, family, molecular and biological functions of the potential candidate genes. Further, the key features of the domain and InterPro annotation were searched in Pfam and Prosite to check the characteristics of the protein. Only those genes with known function and/or related to photosynthesis and metabolic detoxification were considered as potential candidate genes for metribuzin tolerance in wheat.

**Principal component analysis (PCA)**

PCA was performed on the SNP calls of the seven parents to determine genetic relatedness/diversity. SNP alleles were converted to a 1/0 binary system, followed by PCA performed using the built-in R function ‘prcomp’ and data was visualized using the ‘dudi.pca’ function from the ade4 R package [34] using SNP as variables.

**Genetic analyses**

The contribution of maternal or cytoplasmic effects on the differences between population means was assessed by comparing the means of reciprocal F1 crosses. The mode of inheritance of metribuzin tolerance was estimated for each cross combination by generation mean analysis. Mean data on SS recorded on different generations, viz. parents (P1 and P2), F1, F2, BC1 and BC3 for nine cross combinations, were subjected to a scaling (A, B, C and D) and joint-scaling test using the weighted least squares method, which testifies the presence or absence of epistasis [35–37]. When the additive–dominance model fitted the data, a generation variance analysis was performed based on the method described by Allard [38]. This provided estimates of additive and dominance components of variance. The estimated gene effects: mean (m), additive (d) and dominance (d) values were tested by t-test at the 0.05 and 0.01 levels of probability. Further, the goodness-of-fit of the model was tested by comparing expected means of the six generations, calculated from the parameter estimates and observed generation means using chi-squared ($\chi^2$) statistic, and the significance of each parameter was tested using a $t$-test [35, 36].

The nature of dominance was determined from the potency ratio according to [38] $P = \frac{F_1 - M \cdot P}{0.5 (P_1 - P_2)}$, where P is the relative potency of the gene set, F1 is the first generation mean, P1 is the mean of the lower parent, P2 is the mean of the higher parent, and M.P. is the mid-parent value. Complete dominance was indicated when $P = -1$ or $+1$, while partial dominance was indicated when $P'$ was $-1$ or $+1$, except for zero, which indicates the absence of dominance. Over dominance was indicated when the potency ratio exceeded $+1$. The positive and negative signs indicate the direction of the dominance of either parent.

The generalized linear model based on Poisson regression was fitted to the SS data of $F_5$, $F_6$, and $F_7$ RILs from the, Chuan Mai 25 × Ritchie cross using glm() function in R and heritability was calculated based on ANOVA using the formula: $h^2 = \frac{\delta_g^2}{\delta_g^2 + \delta_e^2}$ where $\delta_g^2$ and $\delta_e^2$ are the estimated genotypic and error variances, respectively. The

![Fig. 5 Leaf senescence rating from 0 to 9; plants with an average SS ≤ 3 recorded tolerant (T), 4 to 5 moderately tolerant (MT), and 6 to 9 as susceptible (S)](image-url)
estimated genotypic and error variances were calculated as: $\delta^2_g = \frac{MSg - MSe}{r}$ and $\delta^2_e = \frac{MSe}{r}$, where MSe is the mean square of the RILs, MSe is the residual error and r is the number of replicates. Further, the number of genes controlling metribuzin resistance in each cross was estimated using Wright’s formulae [39, 40].

**Supplementary information**

Supplementary information accompanies this paper at https://doi.org/10.1186/s12871-019-2070-4.

**Additional file 1: Table S1.** The SNPs and their alleles in tolerant and susceptible bulk.

**Abbreviations**

BC$_C$: Backcross of F1 to susceptible parent; BC$_T$: Backcross of F1 to tolerant parent; CM: Chuan Mai 25; CYP: Cytochrome P450; d: Additive gene effect; D: Dagger; ER: Eagle Rock; F: Fundulea 490; F1: First filial generation; F2: Second filial generation; GPX: Glutathione peroxidase; GST: Glutathione S-transferase; GT: Glycosyltransferase; h: Dominance gene effect; K: Kite; MAS: Marker-assisted selection; mp: mid-parent value; PCA: Principal component analysis; PSB: Photosystem II; R: Ritchie; S: Spear; SNP: Single nucleotide polymorphism; SS: Sessencence score

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**Authors’ contributions**

RB, SP, KHMS and GY conceived and designed the experiments; RB conducted the major experiment, and HL and LX assisted in population development and herbicide spraying; RB and HL analyzed the data, RB wrote the manuscript, and HL, SP, GY and KHMS critically reviewed the manuscript. All authors approved the final version of the manuscript.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests for this research.

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