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# Identification of Candidate Genes for English Grain Aphid Resistance from QTLs Using a RIL Population in Wheat

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**Abstract:** The English grain aphid (EGA) (*Sitobion avenae* F.) is one of the most destructive species of aphids in wheat- (*Triticum aestivum* L.) planting areas worldwide. Large quantities of insecticides are usually used to control aphid damage. The identification of new EGA-resistant genes is necessary for sustainable wheat production. The objective of this study was to identify candidate genes for EGA resistance from stable quantitative trait loci (QTLs). We previously constructed a genetic map of unigenes (UG-Map) with 31,445 polymorphic sub-unigenes via the RNA sequencing of 'TN18 × LM6' recombinant inbred lines (TL-RILs). The relative aphid index (RAI) for the TL-RILs was investigated for two growing seasons, with three measured times (MTs) in each season. Using the UG-Map, 43 candidate genes were identified from 22 stable QTLs, with an average of 1.95 candidate genes per QTL. Among the 34 candidate genes annotated in the reference genome Chinese Spring (CS) RefSeq v1.1, the homologous genes of seven candidate genes, *TraesCS1A02G-319900*, *TraesCS1B02G397300*, *TraesCS2D02G460800*, *TraesCS4A02G015600LC*, *TraesCS5B02G329200*, *TraesCS-6A02G000600* and *TraesCS6A02G418600LC* have been previously reported to play roles in aphid resistance. This suggests that these genes are strongly associated with EGA resistance in wheat. The candidate genes in this study should facilitate the cloning of EGA-resistant genes and genetic improvement in wheat breeding programs.

**Keywords:** wheat; English grain aphid (EGA); *Sitobion avenae*; quantitative trait locus (QTL); recombinant inbred line (RIL); candidate gene; genetic map of unigenes (UG-Map); relative aphid index (RAI)



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## 1. Introduction

Aphids are among the most devastating pests to crop production because of their short life cycles and enormous reproductive potential [1,2]. Common wheat (*Triticum aestivum* L.) supplies approximately one-fifth of the calories and protein for human diets [3]. Aphids are the most serious insect pests for wheat. The aphids that severely affect wheat production around the world are the English grain aphid (EGA) (*Sitobion avenae* F.), Russian wheat aphid (RWA) (*Diuraphis noxia* M.), greenbug (*Schizaphis graminam* R.), bird cherry-oat aphid (*Rhopalosiphum padi* L.), etc. [4–6]. Among them, the EGA is one of the most destructive of the aphid species in wheat-planting areas worldwide [7,8]. The EGA mainly feeds on wheat at the jointing and booting stages and causes considerable damage to wheat yield and quality by phloem sap sucking, excreting honeydew and transmitting plant viruses [9–11].

This results in annual wheat yield losses ranging from 15 to 30% and as high as 60% in severe cases [6,12].

Over the long course of their evolution, plants have evolved a variety of adaptations to reduce the damage caused by phytophagous insects. Their mechanisms of resistance against aphids have traditionally been divided into three classic categories: antibiosis, tolerance and antixenosis [13,14]. A resistant cultivar may exhibit a combination of these categories of resistance [15]. Antibiosis is the main resistance mechanism of aphid resistance in plants. Plants produce chemicals that are harmful to aphids or lack the nutrients that are necessary to aphids, resulting in increased aphid mortality and reduced reproductive rate [15,16]. Antibiosis is associated with the nutritional composition and secondary chemicals of wheat such as soluble sugars, amino acids, DIMBOA, alkaloids, and phenolics [4,17]. Tolerance is a characteristic that allows the host plant to grow and develop normally even after aphid infestation, showing a certain regeneration or compensation ability at the individual or population level and not greatly reducing production. This is a passive resistance mechanism that acts through repair or de novo synthesis of photosystem proteins [18]. Antixenosis, or what is referred to as non-preference, involves morphological resistance in which the aphid is either repelled by or not attracted to the host plant, for feeding as well as for egg-laying. For example, the wax powder, glume color and ear density of wheat affect the feeding and reproduction of aphids [19,20].

At present, large quantities of insecticides are usually used to control aphid damage. However, these substances cause serious environmental pollution, threaten human health and destroy non-targeted beneficial insects [21,22]. The use of resistant wheat cultivars can provide an efficient, economic and environmentally friendly approach to reducing the yield losses caused by aphid injury and can produce healthy, pesticide-free products [4,23,24]. Therefore, improving the resistance of host-plants has become a high-priority target in wheat breeding programs. To date, a great number of wheat germplasms have been evaluated for EGA resistance using various methods, and many wheat cultivars have been identified as being resistant to EGA [4,25–28]. Identifying EGA-resistant genes and linked molecular markers is crucial for gene cloning and enhances the efficiency of molecular breeding in wheat. This approach enables precise selection and incorporation of EGA resistance into new wheat varieties. Furthermore, EGA resistance in wheat exhibited a typical quantitative trait inheritance pattern [6]. Quantitative trait locus (QTL) analysis has proved an effective approach to dissect a complicated quantitative trait into component loci and is an effective strategy for crop improvement [29,30].

The wheat EGA resistant genes and their closely linked molecular markers have been reported. Fu et al. [31] assessed the natural infection of EGA in an $F_2$ population and identified the *dnY* gene for EGA resistance on chromosome 7D using SSR markers. Hu [32] artificially inoculated a RIL population in the field and found the *Ra* gene for EGA resistance on chromosome 7D. Wang et al. [24,33] reported the EGA-resistant *Sa1* and *Sa2* genes on chromosome 7D by counting the number of EGA in tillers of $F_1$, $F_2$, $BC_1$, $F_{2:3}$ and $F_{3:4}$ populations under natural infection. Liu et al. [4] found EGA resistance gene *RA-1* on chromosome 6AL of *Triticum durum* through artificial inoculation in field and greenhouse settings using $F_1$, $F_2$ and $F_{2:3}$ populations. Using 70 bread wheat accessions, Li and Peng [34] identified four SSR loci significantly associated with EGA resistance, four with EGA tolerance and four SSR loci were detected continuously at different growing stages of wheat. Luo et al. [26] found five potential expressed sequence tags likely associated with EGA resistance and one was located on 7DL using two backcross progenies.

Some QTLs and/or candidate genes associated with EGA resistance have also been identified. Zeng et al. [35] constructed a genetic linkage map with a total of 14,694 polymorphic SNPs of a RIL population. One major QTL, *QSa3.haust-3D*, was identified for EGA resistance on 3DL between 550.25 and 552.98 Mb in Chinese Spring (CS) RefSeq v1.1 [36], and eight putative candidate genes were identified from this region [36]. Zhao et al. [6] identified a new locus of *qSa-3A* for EGA resistance in a narrow region of 34.52–37.50 Mb on chromosome 3AS. They identified nine important loci and nine QTLs using genome-

wide association study (GWAS) and QTL analysis, respectively, providing the physical intervals in the reference genome CS RefSeq v1.1 [36]. A number of candidate genes were identified by expression analysis and gene-silencing technology. Fu et al. [10] reported that the expression of harpin protein Hpa1 inhibits EGAs from feeding from the phloem. This defensive mechanism was shown as enhanced expression of wheat genes encoding phloem lectin proteins (PP2-A1 and PP2-A2) and β-1,3-glucan synthase-like enzymes (GSL2, GSL10 and GSL12). Luo et al. [37] found that the expression levels of the candidate genes *SaEST1* and *SaEST2*, which are the putative photosystem I assembly protein Ycf3 and vegetative cell wall gp1-like protein, respectively, up-regulated the EGA resistance in flag leaves. Wang et al. [38] identified *lipoxygenase linoleate* (*LOX*) genes, *TaLOX5*, *7*, *10*, *24*, *29*, *33*, specifically expressed post-EGA infestation using RNA-seq data and qRT-PCR analysis. Zhai et al. [39] elucidated that the *TaMYB19*, *TaMYB29* and *TaMYB44* are co-regulators of wheat plant phloem-based defence (PBD) using qRT-PCR and the gene silencing method. Furthermore, a 13-lipoxygenase gene, designated as *TtLOX*, was cloned and functionally characterized. *TtLOX* is involved in wheat resistance against EGA and the regulation of jasmonate biosynthesis [17].

We previously constructed a genetic map of unigenes (UG-Map) via the RNA sequencing of 'TN18 × LM6' recombinant inbred lines (RILs) [40]. The objective of this study was to perform QTL analysis for EGA resistance using this RIL population, and then to identify candidate genes from stable QTLs.

## 2. Materials and Methods

### *2.1. Plant Materials*

A set of 184 RILs derived from the cross of 'TN18 × LM6' using a single-seed descent approach were used to perform QTL analysis [41,42]. For convenience, we named this RIL population as TL-RIL. The female parent TN18 is a variety developed by our group. TN18 has become a core parent, with more than 60 authorized cultivars developed as of 2023. The male parent LM6 is an elite wheat line developed by the Linyi Academy of Agricultural Sciences. The two parents show differences in aphid resistance, with LM6 having higher resistance than TN18 overall.

### *2.2. Experimental Design and Trait Measurement*

The trials were carried out at the Experiment Station of the Shandong Agricultural University (Tai'an, China). The test field was covered at the top and open around the sides. The rainfall during wheat growth was approximately 160 mm, and the soil structure was loamy soil. The compound fertilizer (N-15%, P-15% and K-15%) was applied at 750 kg per ha, watered two times at the jointing stage and flowering stage (600 $m^3$ per ha for one time) under full soil moisture sowing conditions and no pesticides were used. Seeds were sown on 10–11 October, and plants were harvested on 9–10 June the next year.

The TL-RIL population and its parents were planted in a single hole with a spacing of 30 × 30 cm, 10 seeds per hole, five plants reserved at the seedling stage, and two replications in two growing seasons 2019–2020 and 2020–2021. The number of aphids was investigated under natural infestation, with three measured times (MTs) in each year, every 7 days in the jointing stage [35]: 23 April (20MT1), 30 April (20MT2) and 7 May (20MT3) in 2020; and 21 April (21MT1), 28 April (21MT2) and 5 May (21MT3) in 2021. For each replication, the number of EGAs on 20 tillers was measured for each line. The number of EGAs per tiller (NET) was calculated. The relative aphid index (RAI) was calculated via dividing the NET by the average of all NET values [13,24]. The criterion of resistance to the aphid was divided into seven types, including: immune (I), RAI = 0; highly resistant (HR), RAI = 0.01–0.30; moderately resistant (MR), RAI = 0.31–0.60; lowly resistant (LR), RAI = 0.61–0.90; lowly susceptible (LS), RAI = 0.91–1.20; moderately susceptible (MS), RAI = 1.21–1.50; highly susceptible (HS), RAI > 1.51 [4,13,14].

### *2.3. Data Analysis, QTL Detection and Candidate Gene Identification*

Analysis of variance (ANOVA) was performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Genotypes and environments were considered as two factors using the data from 184 lines under six MTs. All factors involved were considered sources of random effects.

The UG-Map for the TL-RILs was used for QTL analysis. Each line of the TL-RILs was sequenced using RNA-Seq technology. The clean reads were mapped to Chinese Spring (CS) RefSeq v1.1 [36]. The polymorphic unigenes were used to construct the UG-Map based on their physical positions. The final UG-Map included 31,445 polymorphic sub-unigenes, which represented 27,983 unigenes and 118,120 SNPs/InDels [40]. The mRNA expression levels for a SNP/InDel of candidate genes were determined by the number of reads mapped on the reference genome for all lines of TN18 or LM6 genotypes in the TL-RILs. When a gene included more than one SNP/InDel, we regarded the expression levels of the gene as different and at the least one SNP/InDel as different.

Windows QTL Cartographer 2.5 software (http://statgen.ncsu.edu/qtlcart/WQTLCart.htm, accessed on 5 January 2019) was used to perform QTL mapping, and composite-interval mapping (CIM) was selected to search for QTLs. The parameter setup was as follows: "Model 6 standard analysis" with a walk speed of 0.5 cM, "forward and backward" regression for the selection of the markers to control for the genetic background, up to five control markers and a blocked window with a size of 10 cM to exclude closely linked control markers at the testing site [42]. The threshold for declaring the presence of a significant QTL in each MT was LOD $\geq$ 3.0, and the QTL interval was determined by LOD $\geq$ 2.0. A QTL was defined as a stable QTL when it was detected over (AV+2MTs).

The unigenes covered by or adjacent to the interval of QTLs were regarded as candidate genes for the corresponding QTLs.

## 3. Results

### *3.1. Phenotypic Variation Analysis*

For the parents of the TL-RIL population, the number of EGAs per tiller (NET) for TN18 were 8.39, 23.40, 28.55, 13.11, 17.09 and 41.84, and were 5.61, 18.72, 29.51, 6.33, 13.04 and 28.62 for LM6 in 20MT1, 20MT2, 20MT3, 21MT1, 21MT2 and 21MT3, respectively. The relative aphid index (RAI) showed differences with 2.00 and 1.58 in average values (AVs) for TN18 and LM6, respectively (Table 1). For the RIL population, the AVs of NET in 20MT1, 20MT2, 20MT3, 21MT1, 21MT2 and 21MT3 were 2.86, 6.96, 14.10, 7.97, 11.40 and 22.87, respectively. The ANOVA showed that the variance for genotype effects on RAI was significant at $p \leq 0.001$ (Table 2). The RIL population exhibited high variations, with RAI values ranging from 0.05 (20MT1) to 4.67 (21MT1) and CVs (coefficient of variation, %) ranging from 50.78% (21MT3) to 95.45% (20MT2) under the six measured times (MTs) and their AV (Table 1). Transgressive segregation was observed for all six MTs and AVs, which segregated the resistance types of MR, LR, LS, MS and HS in AVs according to the criterion of resistance to the aphid [4,13,14]. The RAI examined at each MT displayed a continuous distribution, underscoring their inherent nature as quantitative traits. (Table 1 and Figure S1).

**Table 1.** Phenotypic parameters for relative aphid index (RAI) of the TL-RILs and their parents.

| Trait | Measured Time (MT) | TL-RIL | | | | | Parent | |
|---|---|---|---|---|---|---|---|---|
| | | AV | MIN | MAX | SD | CV | TN18 | LM6 |
| RAI | 20MT1 | 1 | 0.05 | 4.08 | 0.95 | 95.45 | 3.39 | 2.27 |
| | 20MT2 | 1 | 0.13 | 4.88 | 0.87 | 87.27 | 3.69 | 2.95 |
| | 20MT3 | 1 | 0.20 | 3.50 | 0.64 | 64.14 | 1.73 | 1.79 |
| | 21MT1 | 1 | 0.11 | 4.67 | 0.6 | 89.61 | 1.28 | 0.62 |
| | 21MT2 | 1 | 0.14 | 4.08 | 0.77 | 77.38 | 1.13 | 0.86 |
| | 21MT3 | 1 | 0.18 | 2.80 | 0.51 | 50.78 | 1.49 | 1.02 |
| | AV | 1 | 0.34 | 2.83 | 0.48 | 47.89 | 2.00 | 1.58 |

**Table 2.** Analysis of variance for RAI.

| Trait | Source of Variation | Variance | *F* Value | *p* Value |
|---|---|---|---|---|
| RAI | Environment | 0.048 | 0.078 | 0.996 |
| | Genotype | 2.122 | 3.476 | 0.000 |
| | Environment × Genotype | 0.977 | 1.600 | 0.000 |
| | Error | 0.610 | | |

### *3.2. Major Characteristics of Stable QTLs*

Using Windows QTL Cartographer 2.5, a total of 22 stable QTLs for RAI were located. The QTLs were distributed across 14 chromosomes, with one QTL on 1A, 2B, 2D, 3D, 4B, 5D, 6B and 7B; two QTLs on 1B, 2A, 4A, 6A and 7A; and three QTLs on 5B (Tables S1 and 3 and Figure 1). The maximum LOD value for a single QTL under the different MTs was 16.81 (*QRai-7A-4371* in 20MT2). The $R^2$ of a single QTL ranged from 11.58% (*QRai-5B-11778* in AV) to 55.09% (*QRai-4A-371* in 20E2), indicating that they are major QTLs. Five QTLs, *QRai-5B-13413*, *QRai-6A-10852*, *QRai-7A-4371*, *QRai-7A-4371* and *QRai-7B-5888*, showed positive additive effects, with the female parent TN18 increasing the QTL effects. The other 17 QTLs showed negative additive effects, with the male parent LM6 increasing the QTL effects. Two QTLs, *QRai-1B-10819* and *QRai-2A-16206*, were detected under (AV+5MTs); three QTLs, *QRai-1A-8731*, *QRai-2B-14834* and *QRai-2D-6068*, were found under (AV+4MTs); and nine QTLs, *QRai-3D-1844*, *QRai-4A-4567*, *QRai-5B-10270*, *QRai-6A-2681*, *QRai-6A-7415*, *QRai-6B-13587*, *QRai-7A-4310*, *QRai-7A-4371* and *QRai-7B-6084* were located under (AV+3MTs). These 15 QTLs were detected under the AV and at more than half of the MTs, showing that they should be more important. The other seven QTLs were located under (AV+2MTs).

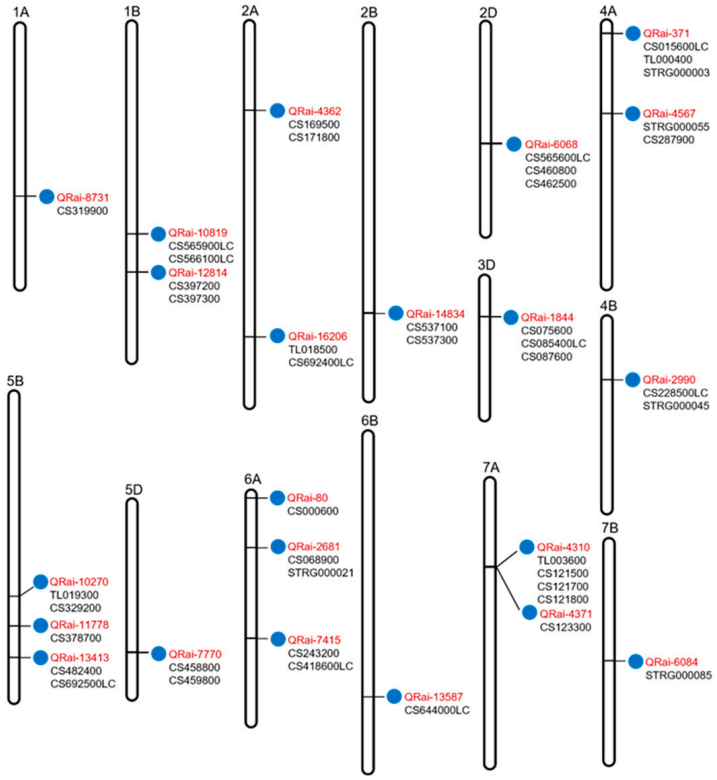


**Figure 1.** Locations of the 22 stable QTLs and their 43 candidate genes for relative aphid index (RAI) using the 'TN18 × LM6' RILs. The red letters are QTLs and the black letters are corresponding candidate genes. CS and TL are abbreviations of gene names of "*TraesCSxx02G*" and "*TraesTLxx02G*".

**Table 3.** Summary for the stable QTLs and their candidate genes for RAI in the TL-RILs.

| QTL | Candidate Gene | Physical Position (bp) | Annotation in RefSeq v1.1 |
|---|---|---|---|
| *QRai-1A-8731* | *TraesCS1A02G319900* | 510,457,220–510,457,634 | Defensin |
| *QRai-1B-10819* | *TraesCS1B02G565900LC* | 55,4225,442–554,227,149 | Non-lysosomal glucosylceramidase |
| □ | *TraesCS1B02G566100LC* | 554,233,155–554,234,043 | Cobalamin-independent synthase family protein |
| *QRai-1B-12814* | *TraesCS1B02G397200* | 628,811,487–628,812,147 | P-loop containing nucleoside triphosphate hydrolases superfamily protein |
| | *TraesCS1B02G397300* | 628,827,507–628,827,752 | Cyclopropane-fatty-acyl-phospholipid synthase |
| *QRai-2A-4362* | *TraesCS2A02G169500* | 124,378,631–124,379,938 | Pentatricopeptide repeat-containing protein |
| | *TraesCS2A02G171800* | 127,202,262–127,205,248 | Small nuclear ribonucleoprotein family protein |
| *QRai-2A-16206* | *TraesTL2A02G018500* | 748,500,956–748,503,630 | No annotation |
| □ | *TraesCS2A02G692400LC* | 748,503,322–748,504,125 | Pentatricopeptide repeat (PPR) superfamily protein |
| *QRai-2B-14834* | *TraesCS2B02G537100* | 732,567,244–732,570,497 | Endo-1,4-beta-xylanase |
| | *TraesCS2B02G537300* | 732,597,482–732,606,603 | Alpha-N-acetylglucosaminidase |
| *QRai-2D-6068* | *TraesCS2D02G565600LC* | 565,586,267–565,598,804 | Polyubiquitin |
| | *TraesCS2D02G460800* | 567,057,496–567,057,801 | Hydroxyproline-rich glycoprotein family protein, putative |
| □ | *TraesCS2D02G462500* | 568,408,975–568,410,748 | Glycosyltransferase |
| *QRai-3D-1844* | *TraesCS3D02G075600* | 36,573,357–36,578,738 | Very-long-chain (3R)-3-hydroxyacyl-CoA dehydratase |
| | *TraesCS3D02G085400LC* | 42,991,128–42,995,699 | Prohibitin |
| | *TraesCS3D02G087600* | 44,195,689–44,198,927 | AT2G31890-like protein |
| *QRai-4A-371* | *TraesCS4A02G015600LC* | 13,558,402–13,560,648 | Auxin-responsive protein |
| | *TraesTL4A02G000400* | 13,558,239–13,560,778 | No annotation |
| □ | *STRG_4A.000003* | 135,58,239–13,561,961 | No annotation |
| *QRai-4A-4567* | *STRG_4A.000055* | 592,926,045–592,927,417 | No annotation |
| | *TraesCS4A02G287900* | 593,113,134–593,116,295 | Peptide transporter |
| *QRai-4B-2990* | *TraesCS4B02G228500LC* | 243,845,832–243,848,118 | Aldolase-type TIM barrel family protein |
| □ | *STRG_4B.000045* | 245,490,724–245,494,619 | No annotation |
| *QRai-5B-10270* | *TraesTL5B02G019300* | 513,323,371–513,324,125 | No annotation |
| | *TraesCS5B02G329200* | 513,325,497–513,326,091 | Defensin |
| *QRai-5B-11778* | *TraesCS5B02G378700* | 556,954,477–556,962,052 | WD-40 repeat protein |
| *QRai-5B-13413* | *TraesCS5B02G482400* | 654,952,573–654,955,232 | DCD (development and cell death) domain protein |
| | *TraesCS5B02G692500LC* | 655,014,971–655,015,377 | Phosphate import ATP-binding protein PstB |
| *QRai-5D-7770* | *TraesCS5D02G458800* | 503,314,962–503,315,225 | ATP synthase subunit b, chloroplastic |
| □ | *TraesCS5D02G459800* | 503,663,583–503,667,476 | Basic helix-loop-helix (bHLH) DNA-binding superfamily protein |
| *QRai-6A-80* | *TraesCS6A02G000600* | 610,042–615,530 | Protein disulfide-isomerase |
| *QRai-6A-2681* | *TraesCS6A02G068900* | 37,407,147–37,408,119 | Peptidyl-prolyl cis-trans isomerase |
| □ | *STRG_6A.000021* | 37,413,294–37,416,768 | No annotation |
| *QRai-6A-7415* | *TraesCS6A02G243200* | 454,706,798–454,708,469 | Pleiotropic drug resistance ABC transporter |
| | *TraesCS6A02G418600LC* | 454,710,314–454,711,118 | F-box family protein |
| *QRai-6B-13587* | *TraesCS6B02G644000LC* | 624,781,544–624,785,394 | Tyrosine-protein phosphatase |
| *QRai-7A-4310* | *TraesTL7A02G003600* | 78,217,517–78,220,408 | No annotation |
| | *TraesCS7A02G121500* | 78,427,689–78,434,616 | TOM1-like protein 2 |
| | *TraesCS7A02G121700* | 78,463,536–78,469,689 | NRT1/PTR family protein 2.2 |
| | *TraesCS7A02G121800* | 78,504,226–78,507,990 | Ribosomal L11 methyltransferase |
| *QRai-7A-4371* | *TraesCS7A02G123300* | 79,640,789–79,643,360 | Late embryogenesis abundant protein |
| *QRai-7B-6084* | *STRG_7B.000085* | 595,646,778–595,647,563 | No annotation |

### *3.3. Candidate Genes Identified from QTLs*

The unigenes covered by or adjacent to the interval of QTLs were defined as candidate genes. As a result, 43 candidate genes (including ncRNAs) for the 22 QTLs were identified (Tables S1 and 3 and Figure 1). The physical positions of the candidate genes were also given in Tables 3 and S1. Among these candidate genes, 34 were annotated in CS RefSeq v1.1 [36] (IWGSC, https://www.wheatgenome.org/resources/annotations, accessed on 13

November 2018) with 24 high-confidence (HC) genes and ten low-confidence (LC) genes, four were annotated in TL-RILs, and five were ncRNAs. The average number of candidate genes per QTL was 1.95 (43/22). Of these, six QTLs, *QRai-1A-8731*, *QRai-5B-11778*, *QRai-6A-80*, *QRai-6A-2681*, *QRai-6B-13587*, *QRai-7A-4371* and *QRai-7B-6084* included only one candidate gene; 12 QTLs, *QRai-1B-10819*, *QRai-1B-12814*, *QRai-2A-4362*, *QRai-2A-16206*, *QRai-2B-14834*, *QRai-2B-14834*, *QRai-4A-4567*, *QRai-4B-2990*, *QRai-5B-10270*, *QRai-5B-11778*, *QRai-5B-13413*, *QRai-5D-7770*, *QRai-6A-2681* and *QRai-6A-7415* contained two candidate genes; and the other four QTLs included 3–4 candidate genes (Table 3 and Figure 1).

### *3.4. Variant Types and Excellent Mutations for the Candidate Genes*

For the 34 candidate genes annotated in CS RefSeq v1.1 [36], 20, 1 and 2 were non-synonymous, UTR and synonymous mutation(s) in exons, respectively, and 11 were mutations in introns (Table S2). Among all the 43 candidate genes, the reads of 29 genes were significantly different between the TN18 and LM6 genotypes in the TL-RILs, indicating that their mRNA expression levels were changed. The mRNA expression levels of the other 14 candidate genes between the TN18 and LM6 genotypes were not different (Table S2). According to the DNA sequences of the parents and a part of RILs, the promoter region (−2000 bp from the start site of the 5′UTR) of 16 candidate genes in CS RefSeq v1.1 [36] and three candidate genes in TL-RILs were mutated (Table S3). For the two genes with synonymous mutation (*TraesCS4A02G287900* and *TraesCS6A02G000600*), mRNA expression levels were all changed, and the promoter region of *TraesCS4A02G287900* was mutated. For the 11 genes with mutations in introns, six (*TraesCS1B02G565900LC*, *TraesCS2D02G565600LC*, *TraesCS5B02G378700*, *TraesCS5D02G458800*, *TraesCS6B02G-644000LC*, *TraesCS7A02G123300*) showed significantly different mRNA expression levels between the TN18 and LM6 genotypes in the TL-RILs, and one (*TraesCS7A02G123300*) was mutated in the promoter region (Tables S2 and 3).

For all 43 candidate genes, the excellent mutations of 33 candidate genes with 82 SNPs/InDels were TN18 type, for which the additive effects of corresponding QTLs were negative, with the parent TN18 decreasing RAI values. Whereas, the excellent mutations of ten candidate genes with 51 SNPs/InDels were LM6 type, for which the QTL effects were positive, with LM6 decreasing RAI values (Table S3).

## 4. Discussion

### *4.1. Candidate Genes in This Study*

In this study, we identified 43 candidate genes with resistance to EGAs on 14 chromosomes. Four resistance genes for EGA, *dnY* [31], *Ra* [32], *Sa1* and *Sa2* [24,33], were previously located on the chromosome 7D. Our investigation did not reveal any candidate genes on this chromosome. The work of Liu et al. [4], which identified *RA-1* closely linked to the SSR markers *Xwmc179*, *Xwmc553* and *Xwmc201* on 6AL, falls outside our findings of four candidate genes on chromosome 6A (Tables 3 and S1). Similarly, the five SSR markers (*Xgwm11*, *Xgwm294*, *Xgwm526b*, *Xgwm192b* and *Xgwm613b*) highlighted by Li and Peng [34] were on the same chromosome as this study, but the physical positions are different.

A number of candidate genes were identified. Luo et al. [26] found five potential candidate genes, which are different from our study. *QSa3.haust-3D* included eight putative candidate genes, *TraesCS3D02G440100*, *TraesCS3D02G440300*, *TraesCS3D02G440400*, *TraesCS3D02G440800*, *TraesCS3D02G441200*, *TraesCS3D02G441300*, *TraesCS3D02G441900* and *TraesCS3D02G443500* [35]. We identified three genes *TraesCS3D02G075600*, *TraesCS-3D02G085400LC* and *TraesCS3D02G087600* on 3D, which are different from the candidate genes of *QSa3.haust-3D*. Some other candidate genes were identified, including *Hpa1* [10], *SaEST1* and *SaEST2* [37], six *TaLOXs* [38], three *TaMYBs* [39], related genes *PP2s* and *GSLs* [10,39] and a gene *TtLOX*, which was cloned [17]. Their annotations are different from the candidate genes in this study. Zhao et al. [6] identified a locus, *BS00053834_51/RAC875_rep_c105150_-1024*, at the physical interval of 750.55–754.36 Mb on chromosome 2A. We identified two candidate genes, *TraesTL2A02G018500* (748,500,956–748,503,630 bp) and

*TraesCS2A02G692400LC* (748,503,322–748,504,125 bp) for *QRai-2A-16206* (Tables 3 and S1), which are 2 Mb upstream of this locus and may be accordant each other. The above comparison indicated that most of candidate genes in this study should be novel.

In yeast, both synonymous and non-synonymous mutations disturbed the levels of mRNA expressions and affected the fitness effects, which negated the presumption that synonymous mutations are neutral or nearly neutral [43]. In this study, we also found that the RNA expression levels were changed for two candidate genes with synonymous mutations and six genes with mutations in introns. Contrastingly, the RNA expression levels were not changed for five genes with intronic mutations, indicating that they might be less reliable candidates.

We identified 23 lines with moderate resistance (RDI ranging from 0.31 to 0.60), which include TL96, TL107, TL146, TL128, TL168, TL160, TL114, TL176, TL28, TL148, TL80, TL120, TL71, TL18, TL16, TL34, TL33, TL35, TL72, TL78, TL112, TL55, TL37 and TL79. These lines should be favorable for the wheat aphid resistance breeding program.

### *4.2. Seven Candidate Genes Have Been Reported to Play Roles in Aphid Resistance*

We used the UG-Map of the TL-RILs that included 31,445 sub-unigenes to identify candidate genes for EGA resistance from QTLs. The number of candidate genes per QTL was 1.95, which can directly identify the candidate genes for EGA resistance from QTLs. Among the 34 candidate genes annotated in CS RefSeq v1.1 [36], the homologous genes of seven candidate genes (7/34 = 20.6%) have been reported to play roles in aphid resistance (Table S4). We infer that these seven genes are strongly associated with EGA resistance in wheat. This result demonstrated that the candidate genes found in this study were highly reliable. As far as we know, only one resistant EGA gene, *TtLOX*, has been isolated in wheat (*Triticum turgidum*) [17]; these highly reliable candidate genes for EGA resistance provided options for further confirmation. The candidate genes in this study need to be validated using transgenic methods such as CRISPR/Cas9 system.

The seven candidate genes, which are strongly associated with EGA resistance, were TraesCS1A02G319900, TraesCS1B02G397300, TraesCS2D02G460800, TraesCS4A02G-015600LC, TraesCS5B02G329200, TraesCS6A02G000600 and TraesCS6A02G418600LC (Table S4). The details of their resistance to aphids and the variation in TL-RILs of the seven genes were as follows.

*TraesCS1A02G319900* for *QRai-1A-8731* and *TraesCS5B02G329200* for *QRai-5B-10270* are annotated as defensins. Plant defensins have a negative effect on phloem-feeding aphids [44]. Overexpression of biologically safe *Rorippa indica* defensin has been found to enhance aphid tolerance in mustard, with the mean number of surviving mustard aphids on the transgenic and control plants were found to be statistically significant [45]. *TraesCS1A02G319900* had three non-synonymous substitutions (Ile37Val, Gly62Asp and Trp69Arg), one synonymous variant in the exon and 16 SNPs and two InDels in the promoter (Tables S2 and S3, Figures S2A, S3A and S4A). *TraesCS5B02G329200* had one non-synonymous substitution (Gly58Ser) and one synonymous variant in the exon, one SNP in the 3′UTR, two variants in the introns and 10 SNPs in the promoter (Tables S2 and S3, Figures S2B, S3B and S4B). For both of the two genes, the expression levels were significantly different between the TN18 and LM6 genotypes of the TL-RILs (Table S2).

*TraesCS1B02G397300* for *QRai-1B-12814* is annotated as a cyclopropane-fatty-acyl-phospholipid synthase (CFAS). In cotton, CFAS was significantly upregulated in the *Aphis gossypii*-damaged plants when compared with undamaged plants [46]. *TraesCS1B02G-397300* had one non-synonymous substitution (His80Arg) in the exon, and eight SNPs in the promoter. The expression levels were significantly different between the TN18 and LM6 genotypes of the TL-RILs (Tables S2 and S3, Figures S2C, S3C and S4C).

*TraesCS2D02G460800* for *QRai-2D-6068* is annotated as a hydroxyproline-rich glycoprotein family protein, putative (HRGP). HRGPs are important structural components of plant cell walls and accumulate in response to infection as an apparent defense mechanism [47,48]. Aphid feeding on the sugarcane-aphid-resistant sorghum line resulted in the

increased expression of genes related to cell wall formation, including the *HRGP* gene [49]. *TraesCS2D02G460800* had one non-frameshift deletion (nine amino acid residue deletions) in the exon and no SNP/InDel in the promoter. The expression levels were significantly different between the TN18 and LM6 genotypes of the TL-RILs (Table S2, Figures S2D and S3D).

*TraesCS4A02G015600LC* for *QRai-4A-371* is annotated as an auxin-responsive protein (ARP). In sorghum, the *ARP* gene involved in processes that halt accumulation of free IAA showed 6.6- and 3.9-fold increases in expression in response to aphid herbivory in resistant and susceptible genotypes, respectively. This suggests that up-regulation of this gene may have triggered a change in the allocation of resources from growth to defense and conferred greater resistance to the aphids [50]. *TraesCS4A02G015600LC* had three SNPs in the intron and no SNP/InDel in the promoter. The expression levels were not significantly different between the TN18 and LM6 genotypes of the TL-RILs (Table S2 and Figure S2E).

*TraesCS6A02G000600* for *QRai-6A-80* is annotated as a protein disulfide-isomerase (PDI). PDIs play a role in the redox regulation of target enzymes and transcription factors [51]. The expression of PDI was significantly different between aphid-resistant and aphid-susceptible wheat plants under aphid attack. Additionally, PDIs may be involved in wheat tolerance to RWA by participating in plant cell protection against oxidative stress [52]. *TraesCS6A02G000600* had two synonymous variants in the exon and no SNP/InDel in the promoter. The expression levels were significantly different between the TN18 and LM6 genotypes of the TL-RILs (Table S2 and Figure S2F).

*TraesCS6A02G418600LC* for *QRai-6A-7415* is annotated as an F-box family protein. In cucumber, the expression level of the gene encoding the F-box family protein was up-regulated after aphid infestation. This indicated that aphid infestation enhanced the expression of the gene, suggesting that they could play an important role in aphid resistance in cucumber [53]. F-box domain-containing proteins play a crucial role during aphid herbivory by fine tuning the ethylene pathway in melon [54]. *TraesCS6A02G418600LC* had two non-synonymous substitutions (Trp24Arg and Ser38Ile) in the exon, and six SNPs in the promoter. The expression levels were not significantly different between the TN18 and LM6 genotypes (Tables S2 and S3, Figures S2G, S3E and S4D).

## 5. Conclusions

We used the UG-Map of the TL-RILs to identify candidate genes for EGA resistance from QTLs at six MTs over two growing seasons. A total of 43 candidate genes for 22 stable QTLs were identified. Most of the candidate genes were newly identified. Interestingly, among the thirty-four candidate genes annotated in the CS RefSeq v1.1 [36], the homologous genes of seven candidate genes have been reported to play roles in aphid resistance. These genes were strongly associated with EGA resistance in wheat, which demonstrated that the candidate genes found in this study were highly reliable. The candidate genes in this study should facilitate the cloning of EGA-resistant genes and improve resistance to English grain aphids in wheat breeding.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14030637/s1, Figure S1: Distribution of relative aphid index (RAI) under different measured times (MTs) and average value (AV). Figure S2: Difference of DNA sequences between TN18 and LM6 for candidate genes have been reported to play roles in aphid resistance. Figure S3: Difference of amino acid sequences between TN18 and LM6 for candidate genes that have been reported to play roles in aphid resistance. Figure S4: Difference of promoter region (−2000 bp from the start site of the 5′UTR) between TN18 and LM6 for candidate genes that have been reported to play roles in aphid resistance. Table S1: Stable QTLs and their candidate genes for RAI in the TL-RILs. Table S2: Mutant types and expression levels of reads for the candidate genes. Table S3: SNPs/InDels in the promoter regions of the candidate genes. Table S4: Homologues of candidate genes previously reported to play roles in aphid resistance.

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