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Field-based screening identifies resistance to Sunn pest (*Eurygaster integriceps*) feeding at vegetative stage in elite wheat genotypes

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Abstract. Sunn pest (*Eurygaster integriceps* Puton) is currently widely distributed in West and Central Asia and Eastern Europe, but has not been found in Australia, Western Europe or North America. Climate warming is known to promote the expansion of its range of distribution, and it is expected that the insect could spread into new territories. Varieties of wheat (*Triticum aestivum*) carrying resistance remain an important component of managing the biosecurity risk of any potential incursion. Previous studies have identified sources of Sunn pest resistance in wheat, but there is little information on the genes that confer the resistance. This research used field-based, artificial infestation cages to evaluate 204 elite wheat varieties for Sunn pest resistance, at Terbol, Lebanon. A significant ($P < 0.001$) difference in resistance was observed among the wheat germplasm, with 19 varieties rated as resistant to moderately resistant and 17 as highly susceptible. Three of the elite varieties showed very little damage, a status similar to that of the resistant check, ICBW-209273. In parallel, the research carried out a genome-wide scan with single-nucleotide polymorphism (SNP) markers to identify chromosome regions and putative genes associated with resistance. Association mapping identified SNP markers with significant associations on chromosomes 2D, 4B and 5B. When these markers were projected onto the wheat population sequencing-based (POPSEQ) reference map, they tended to map close to the location of wheat height-reducing genes. The phenotypic variation explained by the identified markers ranged from 7% to 11%, and collectively, they explained 23.9% of the variation or 45% of the generalised heritability. Marker-trait association was confirmed in two independent, doubled-haploid wheat populations, derived from crosses involving wheat landraces from Afghanistan, where Sunn pest is recognised as an endemic problem. In the two wheat populations, the analyses validated the strong association between wsnp_BF483640B_Ta_2_2 and resistance to Sunn pest damage at the vegetative stage. This study demonstrates existence of genetic resistance to Sunn pest feeding at the vegetative stage in elite wheat germplasm. The study also identified and validated SNP markers that could be useful tools for transfer of resistance into new wheat cultivars.

Additional keywords: genome-wide association study (GWAS), genetic resistance, pre-emptive breeding, reduced-height genes (Rht).

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Introduction

Sunn pest (*Eurygaster integriceps* Puton) is a destructive insect pest of wheat (*Triticum aestivum*) in a wide area of the globe from the Near and Middle East to Eastern and Southern Europe and North Africa (Amiri *et al*. 2016). It is widely distributed in West and Central Asia and Eastern Europe (Radjabi 1994; Gul *et al*. 2006; Karimzadeh *et al*. 2014), but has not been found in Australia, Western Europe or North America (Critchley 1998). Climate warming is known to promote the expansion of its range of distribution (Krupnov 2012), and it is expected that the insect could spread into new territories, which is a concern for global food security. Under current models of global climate change, large parts of the wheat-producing regions of the southern coast of Australia will remain optimally suitable for *E. integriceps*, while Northern Europe and Canada will provide new territories as cold-stress boundaries recede (Aljaryian *et al*. 2016).

Gene deployment as a pre-emptive measure is historically the most effective and environmentally friendly approach to manage bio-security risks from invasive pest species. Sunn pest causes

two types of damage to wheat. Overwintered adults (old generation adults) usually move to wheat fields when the crop is at about tillering stage, and they feed on the leaves and shoots for ~4–6 weeks (Critchley 1998). During this period they mate, lay eggs and then die. Nymphs and new generation adults feed at reproductive stage (spikes). During this feeding, they inject a prolyl endoprotease into the grain, which degrades the vital gluten proteins of wheat (Darkoh *et al*. 2010). This enzyme is not injected at the vegetative stage, but by the nymphs and new generation adults feeding at the reproductive stage. Consequently, two approaches to resistance breeding are advocated: (*i*) genetic crop protection, i.e. of the plant body; and (*ii*) genetic protection of seeds in the process of their formation and maturation (Krupnov 2012). Our research focus is on the first approach, to provide resistance at the vegetative stage, because this is crucial in reducing the Sunn pest population at the reproductive stage (nymphs and new generation adults) when it can cause damage to grain quality by feeding on spikes. There is a positive relationship between nymph and new-generation adult density and kernel damage (Canhilal *et al*. 2005). Therefore, a wheat variety with resistance at vegetative stage would negatively affect Sunn pest development and thus reduce its population density at reproductive stage and the likelihood of severe and economic damage to grains.

Several reports have identified genetic resistance to Sunn pest feeding at the vegetative stage, but the sources of resistance were either wheat synthetics or landraces (El Bouhssini *et al*. 2009, 2013). Although these are an exotic source of genetic variation that breeders can exploit (Ogbonnaya *et al*. 2008), they are often of poor agronomic value, difficult to thresh, and generally tall and low yielding, and they frequently have poor quality (Trethowan and Mujeeb-Kazi 2008). Genetic resistance in elite varieties has the advantage of being used directly by growers, or as parents in breeding programs, without the fear of producing undesirable progeny.

The present study characterised a panel of elite wheat germplasm for their susceptibility to Sunn pest, using field-based, artificial infestation cages at Terbol, Lebanon. In parallel, a genome-wide scan was performed, using high-density single-nucleotide polymorphism (SNP) markers to search for quantitative trait loci (QTLs) that might be linked to Sunn pest resistance.

Materials and methods

Phenotyping

In total, 204 wheat varieties were evaluated in this study (see Supplementary Materials, table 1S, available at the journal's website). The collection represents varieties of historical significance, recently released cultivars, and parents used in breeding programs by the major breeding companies (InterGrain, LongReach, AGT) in Australia.

Phenotyping was conducted at ICARDA's experiment station at Terbol, Lebanon, using a previously developed screening technique (El-Bouhssini *et al*. 2007) in which lines are evaluated for resistance under artificial infestation in the field. Briefly, test entries were planted in hill plots, 10 seeds per hill, with two checks (resistant, ICBW-209273; susceptible, Cham6) every 10 test entries. The experiment was laid out in a randomised complete block design with two replications, and enclosed in mesh cages measuring 6 m by 9 m by 3 m. Plants of each hill were infested with three adults at the time of insect migration to wheat fields, around mid-March. Four weeks after infestation, the test entries were evaluated for vegetative-stage damage from Sunn pest feeding on the following 1–6 rating scale to assess shoot and leaf damage (and plant stunting): 1, no damage and no stunting; 2, 1–5% damage with very little stunting; 3, 6–25% damage with low level of stunting; 4, 26–50% damage with moderate level of stunting; 5, 51–75% damage with high level of stunting; 6, >75% damage with severe stunting. Trait heritability (broad-sense) was calculated using the VHERITABILITY procedure of Genstat (Boer *et al*. 2015).

Genotypic data

We used the gene-based, Australian Wheat 9k iSelect Beadchip data, kindly provided by Dr Matthew Hayden (DEPI, Victorian Centre for AgriBiosciences, Bundoora). The 9k SNP assay was developed by the International Wheat SNP Consortium (Cavanagh *et al*. 2013), and generated 8632 SNP markers. The data were cleaned to retain 2631 non-redundant markers that have allele frequencies >5% and/or <10% missing values. These were placed on a linkage map according to the scaled map positions reported previously (Cavanagh *et al*. 2013).

Population structure and linkage disequilibrium

From the mapped SNP data, an optimised set of tag SNPs was selected using the Tagger function of HAPLOVIEW version 4.1 (Barrett *et al*. 2005), and based on pairwise tagging only, with r^2 threshold $\geq$0.8. Population structure was examined by eigenanalysis in Genstat, with the Tracy-Widom statistic used to determine the number of significant principal components (PCs) (Boer *et al*. 2015).

We analysed the extent of linkage disequilibrium (LD) present in the population, because it is the key starting point to QTL detection when using the association mapping approach. LD was calculated as the r^2 (square of the correlation coefficients) between alleles at a pair of marker loci, using the Genstat software. The analyses used information on population structure as a covariate in order to give a more realistic measure of its decay (Boer *et al*. 2015). The decay in LD was investigated by plotting pair-wise r^2 values against genetic distance (cM) between markers on the same chromosome. To establish a baseline for the decay, the 95th percentile of the distribution for unlinked markers (>50 cM) was calculated (Breseghello and Sorrells 2006). The baseline was used to examine trends in LD decay over distance, using a 1-cM moving-means approach (Robbins *et al*. 2011).

Association analysis

Association analysis was performed with Genstat, using the mixed-model framework in which markers were fitted as fixed and genotypes as random (Malosetti *et al*. 2007). The analyses examined two models for the control of false positives: use of PCs to account for population structure (Patterson *et al*. 2006), and use of kinship matrix with coefficients of co-ancestry between genotypes to account for genetic relatedness (Yu *et al*. 2006). We also fitted the naïve model, in which no corrections were made for population structure or relatedness,

and the quantile–quantile plots (QQplot) were used to check for any systematic departure from the null expectation of uniform *P*-values. To adjust for multiple comparisons, the effective marker-matrix dimension method was used to determine threshold for identifying significant associations (Boer *et al*. 2015). Significant SNP markers were subsequently allocated to chromosome arms by BLASTn search of the URGI wheat database (https://urgi.versailles.inra.fr/blast/).

Marker validation

The SNP marker with the strongest link to Sunn pest resistance was tested for confirmation across two doubled-haploid wheat populations. The populations were screened for resistance to Sunn pest at the vegetative stage, under cages at the experiment station at Terbol, by using the same methods as for the mapping populations. Marker-trait associations were analysed using the univariate analysis of variance in Minitab 16 (Minitab, State College, PA, USA).

Results

Phenotypic variation

A significant ($P < 0.001$) difference was observed among the elite wheat accessions for Sunn pest resistance at the vegetative stage of growth, with the generalised heritability estimated as 0.53. A summary of the phenotypic variation is presented in Fig. 1, with the majority (82%) of the accessions showing 26–50% damage, indicating a moderate level of stunting. Cham6, which was used as the susceptible check, showed a high level of stunting, with 51–75% damage. The resistant check (ICBW-209273), on the other hand, showed very little stunting, and limited damage (1–5%) from Sunn pest feeding. The results of phenotyping are presented as supplementary information (for full results, see Supplementary Material, table 1S), and showed that 17 of the 204 elite wheat varieties were highly susceptible, and 19 were rated as resistant to moderately resistant, and among these, three were found to exhibit resistance status similar to that of ICBW-209273 (Table 1).

Genetic properties of the wheat genotypes

With significant genetic variation for Sunn pest resistance in the elite wheat varieties, a genome-wide scan was undertaken to identify associated genetic loci and their chromosomal locations. First, we examined the population for stratification, a factor that can induce false positive results, using the method of eigenanalysis (Patterson *et al*. 2006). Based on the Tracy–Widom statistic, the first four PCs were significant, indicating that the population was not genetically homogeneous. The four PCs accounted for 23.4% of the molecular variance in the panel. The PCs were used to cluster the wheat varieties into

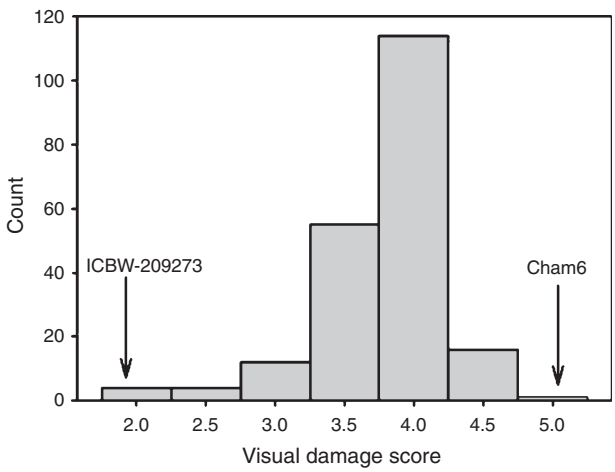

Fig. 1. Phenotypic distribution of mean visual damage scores from two replications obtained for Sunn pest feeding at vegetative stage of growth. The screening was carried out under artificial infestation in the field, with ICBW-209273 as the resistant check and Cham6 as the susceptible check.

Table 1. A representative list of Sunn pest resistance status in elite wheat genotypes screened at the vegetative stage

Wheat variety	Year of release	Damage rating	Status	Pedigree
Dirk	1951	2.00	Resistant	Ford/Dundee
Halberd	1969	2.00	Resistant	Scimitar/Kenya-C-6042//Bobin/3/Insignia-49
Wedin	2010	2.00	Resistant	?
Fronteira	1934	2.50	Mod. resistant	Polyssu/Alfredo-Chaves-6
Gamenya	1958	2.50	Mod. resistant	Kenya-117-A/2*Gabo//Mentana/6*Gabo
GBA Hunter	2005	2.50	Mod. resistant	Attila//Altar-84/Araos/3/Attila
Arnhem	1996	4.50	Susceptible	Pitic-62/Hartog
Batavia	1991	4.50	Susceptible	Brochis(Sib)/Banks
Calingiri	1997	4.50	Susceptible	Chino/Kulin//Reeves
Cascades	1994	4.50	Susceptible	Aroona*3//(Ausen-Vii-95)Tadorna/Inia-66
EGA Wentworth	2004	4.50	Susceptible	Janz*2/Vulcan
Ellison	2003	4.50	Susceptible	Vicam-71/3*Suneca//Sun-231-A
Lang	2000	4.50	Susceptible	Qt-3765/Sunco
Crusader	2008	4.50	Susceptible	Dh)Sunbrook/H-45
Machete	1985	4.50	Susceptible	Mec-3/2*Gabo(Rac-177)//Madden
Stylet	2001	4.50	Susceptible	Molineux/2*Trident,Aus
Sunbrook	1995	4.50	Susceptible	Hartog*2/Suneca; Hartog/Suneca
IG209237 (res. check)	?	2.00	Resistant	?
Cham6 (susc. check)	?	5.00	Highly susceptible	?

subpopulations (Fig. 2). Groups 1 and 3 consisted of a mixture of varieties with different geographic origins, but groups 2 and 4 were exclusively wheat varieties of Australian origin (Fig. 2).

The extent of LD present in the population was then investigated by using an optimised set of 423 tag SNPs. These covered a distance of 2860 cM, with 177 469 pair-wise r^2 values across the hexaploid wheat genomes. The trend of decay with distance is shown in Fig. 3. The baseline value of 0.06 is the estimate beyond which LD is likely to be caused by genetic linkage. The relationship between the baseline and the genetic distance was fitted by using a 1-cM moving means method, and intersected at 17 cM (Fig. 3). This implied that, in the present mapping panel, the LD extended up to 17 cM.

SNP markers associated with Sunn pest resistance

The extent of LD decay implies that a minimum of one marker every 17 cM would be required for genome-wide scan to detect QTLs. We used 5160 SNP markers, and based on the scaled map position of Cavanagh *et al*. (2013), this covered a distance of

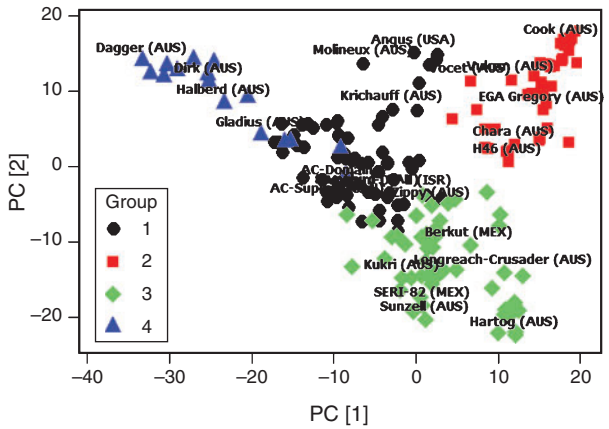

Fig. 2. Population structure of mapping panel analysed by eigenanalysis. Four significant principal component scores identified from Tracy–Widom statistic were used to cluster varieties into groups, based on Ward's linkage and the squared Euclidean distance (Ward 1963).

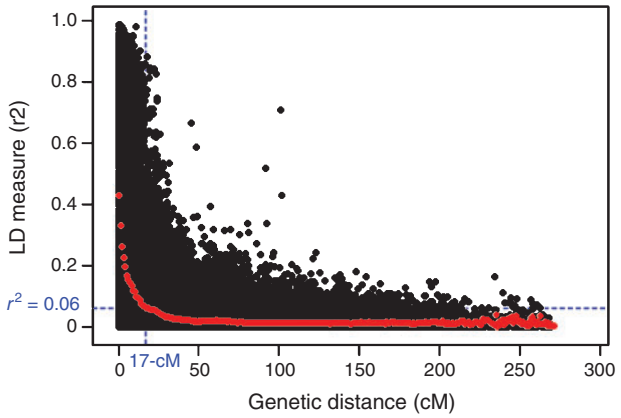

Fig. 3. A graphical plot of the genome-wide linkage disequilibrium (r^2), and its decline with genetic distance. Genetic distance was the scaled map position of Cavanagh *et al*. (2013). Graph displays scatterplot of intra-chromosome r^2 for pairs of SNP markers, after adjusting for population structure. The moving mean r^2 values plotted against distances (red curve) intersected the baseline (the horizontal, dashed blue line) at 17 cM.

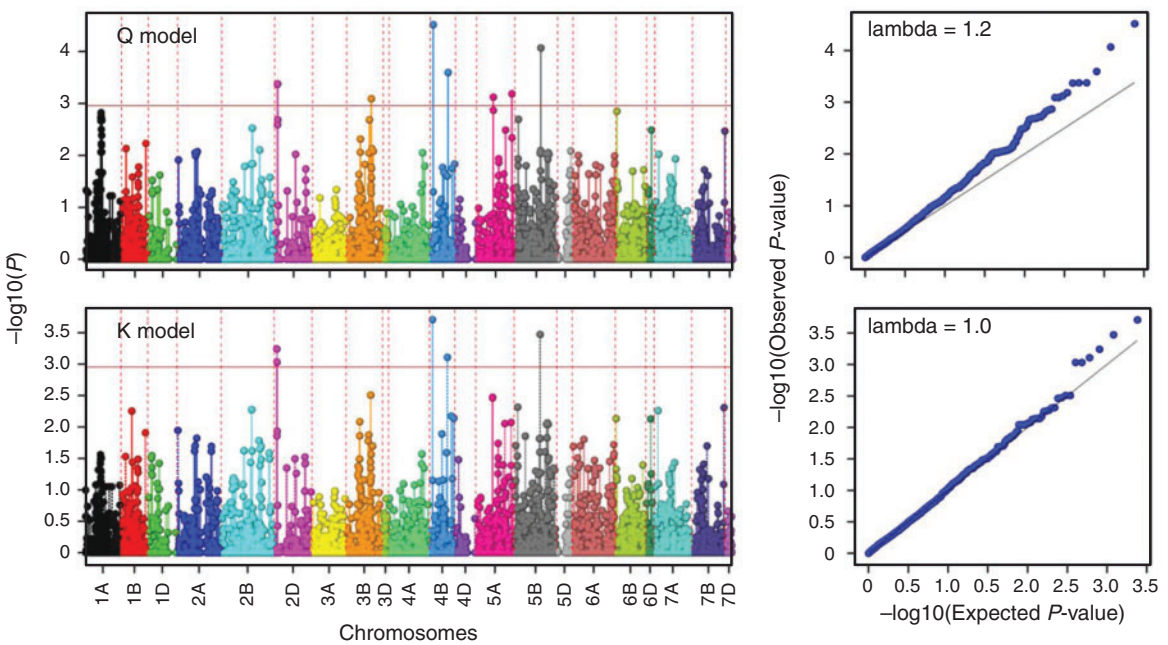

Fig. 4. Manhattan plots of genome-wide scan for genetic loci associated with Sunn pest resistance in commercial wheat. Right side are the quantile–quantile plots to assess effectiveness of different analytical models to control false positives. The horizontal red line represents the significance threshold of –log10(P) = 2.96, above which the null hypothesis of no marker effect was rejected.

Table 2. List of significant SNP markers associated with Sunn pest feeding at vegetative stage, after correcting for genetic relatedness (K model) among elite wheat varieties

Positions are based on the Chromosome Survey Sequence (CSS) contig position on the reference popseq wheat map (https://urgi.versailles.inra.fr/download/iwgsc/POPSEQ/)

SNP name	Alleles	Chr.	Position (cM)	–log10*P*	Allele effects	R^2 (%)
wsnp_Ex_c12250_19568265	[A/C]	2D	8.0	3.03	–0.28 ± 0.09	7.40
wsnp_Ex_c101374_86744680	[A/G]	2D	9.1	3.24	–0.18 ± 0.05	7.28
wsnp_Ex_c7669_13090850	[T/C]	2D	9.1	3.03	–0.28 ± 0.09	7.40
wsnp_BF483640B_Ta_2_2	[T/C]	4B	11.0	3.71	–0.16 ± 0.04	10.76
wsnp_BE499546B_Ta_2_1	[A/G]	4B	87.1	3.11	–0.27 ± 0.08	7.40
wsnp_Ex_c20440_29511162	[T/C]	5B	45.6	3.47	–0.14 ± 0.04	7.42

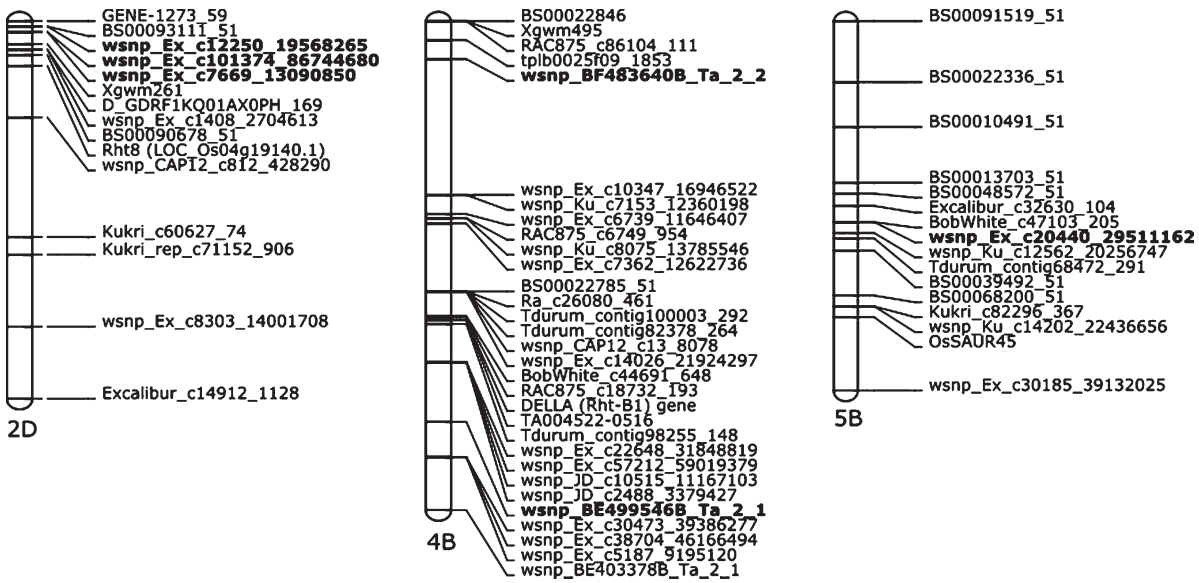

Fig. 5. Map position of markers identified for Sunn pest resistance in genome-wide association analyses (in **bold**). Chromosome maps were based on SNP markers utilised in this study and markers closely linked to *Rht* genes in various studies. These were positioned according to their Chromosome Survey Sequence (CSS) contig position (in cM) on the reference popseq wheat map (https://urgi.versailles.inra.fr/download/iwgsc/POPSEQ/). The SSR markers were projected on the basis of closely linked SNP markers, and positions of *Rht* genes were added to the graph based on their contig position on the popseq map.

3334 cM of the hexaploid genome, providing us with a coverage of one marker every 1.5 cM. This suggests that the marker coverage used for this study was appropriate for detecting QTLs when using a genome-wide association mapping (GWAS) approach.

Manhattan plots from genome-wide analyses are presented in Fig. 4. Initially, we fitted a mixed linear model in which the four significant PCs were used as random factors to account for population substructure within the mapping panel (Q model). As shown in the accompanying QQplot, however, the genomic inflation factor for this model was above unity, which indicates that the observed *P*-value distribution did not closely match expectations (Fig. 4). The use of a kinship matrix to control false positives (K model), on the other hand, resulted in the observed *P*-values closely adhering to the expectation (Fig. 4), indicating no systematic spurious associations.

Using the EXACT method in GENSTAT, the kinship model identified SNP markers on chromosomes 2D, 4B and 5B that satisfied the *P*-value ≤0.001 (–log10(*P*) ≥2.96) cut-off threshold for significance (Fig. 4). The associated loci explained 7–11% of the phenotypic variation (Table 2), and collectively, they explained 23.9% of the variation or 45% of the generalised heritability. The SNP markers were projected onto the wheat population sequencing-based (POPSEQ) reference map (Chapman *et al*. 2015) by using their Chromosome Survey Sequence (CSS) contig positions, and as shown in Fig. 5, the markers tended to map close to regions harbouring wheat genes for reduced plant height. The strongest association was with wsnp_BF483640B_Ta_2_2, a SNP marker on the short arm of wheat chromosome 4B (4BS_4944346). BLASTn analysis of the rice genome identified the putative orthologue to be LOC_Os03 g45180.1 (Chr3; 2e-53), but no specific gene function was found.

Marker validation

For marker validation, we focused on wsnp_BF483640B_Ta_2_2, because it was the most significant and therefore the marker with the closest link to Sunn pest resistance. In the two

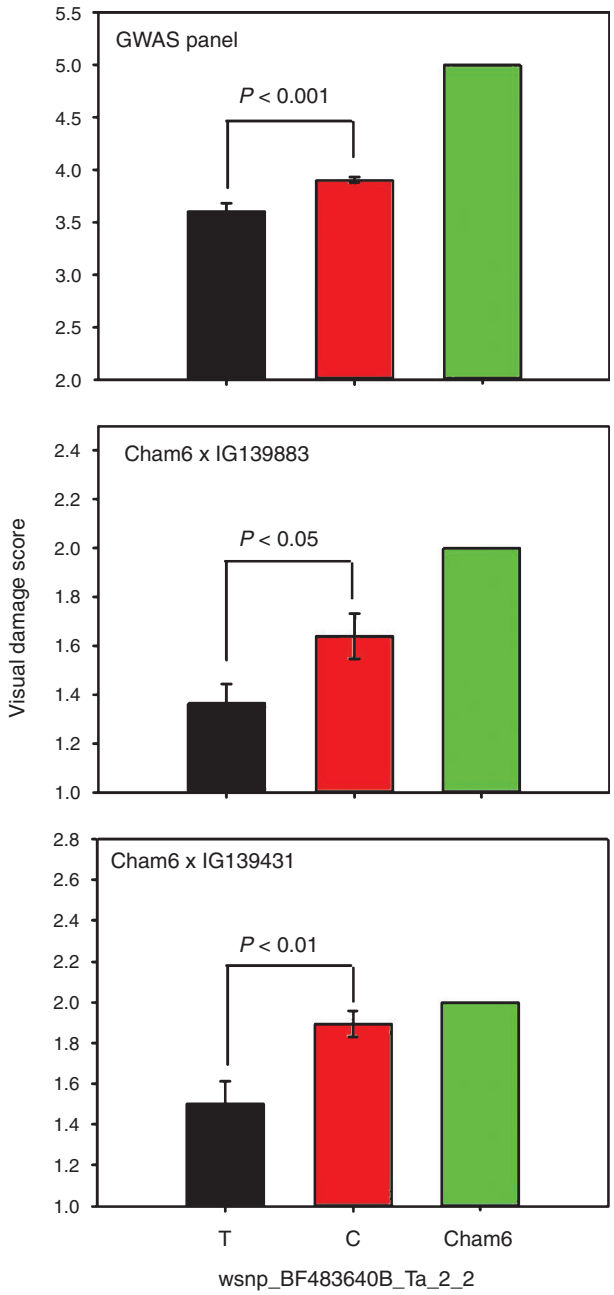

Fig. 6. QTL validation based on the marker with the strongest association to the phenotype. Data from two wheat doubled haploid (DH) populations were used to validate QTL effect. Graph shows allele effects in the original genome-wide association study (GWAS) panel, and across the DH wheat populations.

wheat populations examined, the analyses validated the strong association between wsnp_BF483640B_Ta_2_2 and resistance to Sunn pest damage at vegetative stage (Fig. 6). The 'T' allele was associated with Sunn pest resistance in the original GWAS panel. The QTL effect was still significantly associated with Sunn pest resistance in the two independent populations (Fig. 6), which implies that the marker would be suitable for tagging Sunn pest resistance in breeding programs, irrespective of genetic materials in the crossing block.

Discussion

This study demonstrates existence of genetic resistance to Sunn pest feeding at the vegetative stage in elite wheat varieties. For integrated pest management, use of wheat cultivars carrying resistance at vegetative stage is desirable to reduce the insect population that develop into new-generation adults, which is the most damaging stage for Sunn pest (Canhilal *et al*. 2005). Sunn pest damage at the vegetative stage is influenced by population density of the insect, weather conditions and water availability (Kinaci *et al*. 1998). Thus, resistance would be expected to originate from geographical areas where the climatic conditions favour high population densities of the pest and where selection pressure has enabled the evolution of adaptive genes. It was therefore surprising to identify Sunn pest resistance in wheat genotypes originating from Australia (Table 1) where the insect does not exist. The three resistant genotypes identified in the present study probably possess some specific traits or metabolites that deterred Sunn pest herbivory or enhanced recovery from damage, but their identity is unclear at this stage.

Resistance to Sunn pest feeding at vegetative stage is moderately heritable, as observed in this study ($h^2 = 0.53$). By comparison, Fatehi *et al*. (2009) reported estimates of 0.78–0.84 for broad-sense heritability of resistance to grain damage by Sunn pest. Thus, selecting for resistance at the vegetative stage will not be straightforward, particularly during the early generations of a pre-emptive breeding program. The key challenge, therefore, is the identification of molecular markers that could be used in marker-assisted selection, but to our knowledge, only two reports have been published in this regard (Joukhadar *et al*. 2013; Emebiri *et al*. 2017). The latter study used two bi-parental populations of doubled-haploid lines, and identified a single, consensus QTL on chromosome 4B. The former study used a GWAS mapping approach to associate Sunn pest resistance with DArT markers on 1A, 1B, 2B, 3AS, 4BL, 5AL and 6BS. We also used the GWAS mapping approach, but with a high-density of SNP markers. LD in the GWAS panel extended up 17 cM, and with the high-density marker coverage (one marker every 1.5 cM), these allowed the successful detection of significant QTLs on chromosomes 2D, 4B and 5B (Fig. 4, Table 2). The QTL on chromosome 4B was located in the vicinity of the major locus identified by Emebiri *et al*. (2017), which in itself provided a confirmation of the GWAS results. Further validations (Fig. 6) were obtained from analyses of two independent, doubled-haploid lines derived from crosses involving wheat landraces from Afghanistan, where Sunn pest is recognised as an endemic problem (M. El Bouhssini, pers. obs.).

Is Sunn pest resistance associated with plant height?

An interesting observation regarding the QTLs identified in this study is the close proximity of their map locations to *Reduced-height* genes (*Rht*) in wheat (Fig. 5). The genomic location on chromosome 2D mapped to the short arm of the chromosome where the *Rht8* gene is located. The locus detected on the short arm of chromosome 4B is close to the location of *Rht-B1* gene, as inferred recently by Emebiri *et al*. (2017), and the locus on chromosome 5B is orthologous to the Rice LOC_Os09 g32320 gene, which encodes a growth-regulator related protein. Plant height was not measured in the present study, but Sanaey and

Mirak (2012) reported a highly significant negative correlation between plant height and insect density per m^2, with the authors suggesting that the overwintered Sunn pest adults seem to prefer the short-stemmed wheat for feeding.

Some SNP markers closely linked to height-reducing genes in wheat were polymorphic in our GWAS panel, but they were not found to be associated with Sunn pest resistance, perhaps due to the stringency required to control false positives. When re-analysed with no covariates, the SNP marker wsnp_Ex_c14026_21924297, which was linked to the *Rht-B1* gene at 0.8 cM (Shirdelmoghanloo *et al*. 2016), was significant ($P = 0.03$). Mean comparison at the locus suggested that varieties carrying the dwarfing 'C' allele were more susceptible to Sunn pest damage at the vegetative stage than those carrying the alternative 'T' allele. Varietal allelic composition at the SNP marker is provided as supplementary information (Supplementary material, table 1S). In addition, molecular data were obtained for a subset of the GWAS varieties by competitive allele-specific PCR (KASP; LGC, London) assays for *Rht-B1* and *Rht-D1* markers. Single-marker analysis with these data produced a similar result (as shown in Supplementary material, fig. 1S); that is, wheat varieties carrying the dwarfing alleles were more susceptible, on average, to Sunn pest damage than those carrying the allele for tall height.

Although semi-dwarfing genes reduce plant height in wheat and they were central to enhanced crop yields achieved as part of the green revolution (Hedden 2003), they also have a range of undesirable agronomic effects (Ellis *et al*. 2004). In particular, the *Rht-B1* and *Rht-D1* genes encode truncated DELLA proteins that constitutively restrain plant growth by decreasing sensitivity to endogenous gibberellin hormone level. It is well documented that this has undesirable pleiotropic effects in wheat, such as increased susceptibility to pathogens (Saville *et al*. 2012), poor stand establishment and reduced early seedling vigour (Allan 1980; Addisu *et al*. 2009). In the case of Sunn pest, it is conceivable that the poor early vigour of *Rht* lines could increase vulnerability of the seedlings to attack by overwintered Sunn pest adults. Interestingly, the SNP markers identified on chromosome 2D and 4B for Sunn pest resistance in this study (Fig. 5) are positioned within the chromosomal location of QTLs for early vigour in wheat (*Xgwm261* and *Xgwm495*, respectively) (Rebetzke *et al*. 2001). There are now near-isogenic lines of the *Rht* genes, and these would be used for further research to resolve the functional relationship between Sunn pest resistance and plant height.

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