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ORIGINAL ARTICLE

Effects of *Rht-B1* and *Ppd-D1* loci on pollinator traits in wheat

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Abstract

Key message **Elite wheat pollinators are critical for successful hybrid breeding. We identified *Rht-B1* and *Ppd-D1* loci affecting multiple pollinator traits and therefore represent major targets for improving hybrid seed production.**

Abstract Hybrid breeding has a great potential to significantly boost wheat yields. Ideal male pollinators would be taller in stature, contain many spikelets well-spaced along the spike and exhibit high extrusion of large anthers. Most importantly, flowering time would match with that of the female parent. Available genetic resources for developing an elite wheat pollinator are limited, and the genetic basis for many of these traits is largely unknown. Here, we report on the genetic analysis of pollinator traits using biparental mapping populations. We identified two anther extrusion QTLs of medium effect, one on chromosome 1BL and the other on 4BS coinciding with the semi-dwarfing *Rht-B1* locus. The effect of *Rht-B1* alleles on anther extrusion is genotype dependent, while tall plant *Rht-B1a* allele is consistently associated with large anthers. Multiple QTLs were identified at the *Ppd-D1* locus for anther length, spikelet number and spike length, with the photoperiod-sensitive *Ppd-D1b* allele associated with favourable pollinator traits in the populations studied. We also demonstrated that homeoloci, *Rht-D1* and *Ppd-B1*, influence anther length among other traits. These results suggest that combinations of *Rht-B1* and *Ppd-D1* alleles control multiple pollinator traits and should be major targets of hybrid wheat breeding programs.

Introduction

Bread wheat (*Triticum aestivum* L.) is an important staple crop for human nutrition and is the third most produced food crop after maize and rice (FAO 2015). To meet rapidly rising world population and future food demands, its production needs to increase. This will require substantial changes in agronomic processes as well as technological advances in crop improvement (Tester and Langridge 2010). Hybrid breeding and the ability to exploit heterosis is one of a few high-priority wheat breeding strategies that has the potential to rapidly improve yield and its stability (Longin et al. 2012; Okada and Whitford 2019; Whitford et al. 2013). In a recent study, yield improvements associated with hybrid vigour were demonstrated to be in the order of 10% increase in grain yield as well as improved resistance against biotic and abiotic stresses (Longin et al. 2013). However, wheat is a highly autogamous plant and has accumulated cleistogamous (closed) flower modifications since domestication. For efficient F_1 hybrid wheat seed production, it is important that the male parent is a good pollinator. Ideally, this male parent would be taller than the female parent, exhibit high

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anther extrusion and have large anthers containing abundant long-life pollen that can be easily dispersed via wind over long distances (Whitford et al. 2013). A large spike containing many well-spaced spikelets would facilitate ease in flower opening as well as pollen dispersal. Most importantly, male pollinators should extrude and dehisce their anthers synchronously with female stigma exertion and receptivity; with pollinators having a prolonged flowering duration (e.g. high tillering). All of these traits, therefore, increase the opportunity for cross-pollination. However, in modern wheat varieties, the genetic resources for elite pollinators are currently limited (Langer et al. 2014; Nguyen et al. 2015).

The genetic basis of several key pollinator traits has been extensively studied, including plant height and flowering time. Semi-dwarfing loci *Rht-B1*/*Rht-D1* were introduced into modern wheats throughout the world resulting in the "Green Revolution" as this trait reduced lodging as well as improving harvest index (Pearce et al. 2011; Peng et al. 1999). The photoperiod sensitivity gene *Ppd-1* (Beales et al. 2007; Shaw et al. 2012; Turner et al. 2005) and vernalisation-requirement genes *Vrn1* to *Vrn4* (Kneipp 2017; Yan et al. 2003, 2004, 2006), each contribute towards controlling flowering time under different environmental conditions. Their influence on plant growth and flowering and how they can be utilised for crop improvements has been extensively studied (Guo et al. 2010; Rebetzke et al. 2007; Wilhelm et al. 2013; Zhang et al. 2008). In addition to plant height and flowering time, anther extrusion (AE) is another pollinator trait that has been extensively studied to unravel the underlying genetic basis. Over the last few years, several studies have revealed that AE is controlled by multiple loci of low to medium effect (Boeven et al. 2016; Buerstmayr and Buerstmayr 2015, 2016; He et al. 2016a, b; Lu et al. 2013; Muqaddasi et al. 2016, 2017a, b; Skinnes et al. 2010; Würschum et al. 2018). Several of these studies showed that *Rht-B1*/*Rht-D1* loci have a medium effect, with the semi-dwarfing alleles (*Rht-B1b*/*Rht-D1b*) decreasing AE and increasing anther retention (Boeven et al. 2016; Buerstmayr and Buerstmayr 2016; He et al. 2016b; Muqaddasi et al. 2017b). In contrast, a few other studies using genome-wide association study (GWAS) and a biparental mapping population reported no significant association of *Rht-B1*/*Rht-D1* loci with AE (He et al. 2016a; Muqaddasi et al. 2016, 2017a). This was partly due to a low number of *Rht-B1*/*Rht-D1* lines in the GWAS mapping population (Muqaddasi et al. 2016, 2017a), but it can also be interpreted as *Rht-B1*/*Rht-D1* loci affecting AE only in certain genetic backgrounds. Indeed, Würschum et al. (2018) reported that some of the highest AE pollinator lines carry the semi-dwarfing alleles *Rht-B1b* or *Rht-D1b* (Würschum et al. 2018). Thus, genetic association between AE and *Rht-B1*/*Rht-D1* needs further study. Anther length (AL) is also an important pollinator trait and has been shown to have a significant positive correlation with anther extrusion, pollen grain number per anther and pollen mass (Langer et al. 2014; Milohnic and Jost 1970; Nguyen et al. 2015). However, the genetic basis for AL in wheat is limited (Song et al. 2018), especially that contributing to larger size. This holds true for model species such as rice and Arabidopsis, with the exception of reduced anther size often being associated with male sterility (e.g. Binghua and Jingyang 1986; Cheng et al. 2006; Sasakuma et al. 1978). Furthermore, no single genetic locus has been described influencing multiple pollinator traits in wheat, which in turn could play a major role in facilitating cross-pollination for hybrid wheat seed production.

This study aimed at understanding the genetic basis of wheat pollinator traits using both biparental mapping populations and near isogenic lines (NILs). Genetic analysis of two F_2 populations identified two AE QTLs, one on chromosome 1B, while the other coincides with the *Rht-B1* locus on chromosome 4B. Both loci have a medium effect on AE. The effect of the *Rht-B1* locus on AE appears to be genotype dependent and is supported by data derived from several mapping populations and NILs. This study also showed that the *Ppd-D1* locus on chromosome 2D is associated with multiple pollinator traits, including AL, spike length (SL), spikelet number (SN) and number of days to heading (DH). Both *Rht-B1* and *Ppd-D1* loci revealed a significant and consistent effect on AL, which is positively correlated with AE. These results suggest that *Rht-B1* and *Ppd-D1* are major effect loci and particular allele combinations can be used for selecting a suite of pollinator traits for successful hybrid wheat seed production.

Materials and methods

Plant materials, growth conditions and experimental designs

The bread wheat (*T. aestivum* L. em Thell) spring-type inbred lines, cid423295sid45 (CIMMYT line: PARUS/3/CHEN/AE.SQ//2*OPATA), Glenwari (GRIS Accession No. K-44557), cid388412sid46 (CIMMYT line: PASTOR//SRMA/TUI/3/SAAR), Gamenya (GRIS Accession No. K-44556) and H1621 (Afghanistan landrace), were selected from spring wheat varieties by pre-screening of AE trait and used to generate F_2 mapping populations (Table 1). All experiments were performed in the same glasshouse facility at the University of Adelaide with temperatures ranging from approximately 15 °C (night) to 25 °C (day) and daylight ranging from 12 h (August) to 14.5 h (January). Plants were grown one per pot in 1800 cm^3 of coco peat soil, with nine pots placed in a bunding tray. Bunding trays were placed in a two row by five column format (ten trays) on each available bench (Fig. S1). For control and adjustment

Table 1 Summary of F_2 mapping populations developed and used in this study

Population	Female parent	Male parent	Dates grown	No. F_2 used
#1	cid423295sid45	Glenwari	Aug 2014–Jan 2015	213
#2	cid388412sid46	H1621	Aug 2014–Jan 2015	215
			Aug 2016–Jan 2017	109
#3	cid388412sid46	Gamenya	Aug 2016–Jan 2017	109
#4	cid388412sid46	Glenwari	Aug 2016–Jan 2017	110

of measurement data, several plants of each parent of the mapping populations were grown and randomly arrayed across all benches. Mapping populations #1 and #2, which were used for QTL analysis (including ~ 215 F_2 plants for each population and ten plants of each parent), were grown between August 2014 and January 2015. Populations #3 and #4, along with additional lines of population #2 (~ 110 F_2 plants for each population), were grown between August 2016 and January 2017 to investigate association of *Rht-B1* genotype with anther extrusion and other pollinator traits. Most of the near isogenic lines (NILs) were obtained from the Australian Grains Genebank (http://www.seedpartnership.org.au/associates/agg): *Rht-B1* in recurrent parent cultivars April Bearded and Mercia carrying *Rht-B1a* allele (Peng et al. 1999; Youssefian et al. 1992), *Rht-D1* in recurrent parent cultivars April Bearded, APD0, Huntsman, Mercia and Nainari carrying *Rht-D1a* allele (Manske et al. 2002; Peng et al. 1999; Richards 1992; Youssefian et al. 1992), NILs for *Ppd-B1* and *Ppd-D1* in recurrent parent cultivar Haruhikari carrying *Ppd-B1b* and *Ppd-D1b* alleles (Tanio and Kato 2007). Five plants of each NIL and recurrent parent were grown between August 2017 and January 2018 in a randomised design.

Phenotyping and measurement of traits

Generally, six pollinator traits associated with cross-pollination efficiency and two additional traits, partly involved in crossability efficiency, were measured for QTL analysis and marker-trait association studies in F_2 mapping populations and NILs. For all traits, the first three spikes of each plant were used for measurements in all experiments and averaged measured value of three spikes was used for QTL analysis. Each spike was tagged at the day of Zadoks scale 57 (Zadoks et al. 1974) with colours used to identify spike emergence order. The following procedures were used to measure the traits: (1) Number of days to heading (DH) were calculated from the date of sowing to the date at Zadoks scale 57 (75% ear emerged from flag leaf), representing flowering time as plants normally initiated flowering 3–4 days after this stage. (2) Visual anther extrusion (AE) was assessed using a scale (0: no AE to 5: high AE) approximately 7 days post anthesis. Each of three spikes was scored independently, according to the flowering time of individual spikes. To ensure consistency and to minimise variation, the same individuals performed all AE trait measurements for all experiments. (3) Anther length (AL) was measured by the following procedure: anthers from primary and secondary florets of two spikelets located at the middle of spike were collected at Zadoks scale 59 (full heading stage) and then stored in a 1.5 mL Eppendorf tube with 70% ethanol at 4 °C. Anther images were subsequently taken using a stereo dissecting microscope Leica MZFL III equipped with the digital camera DFC300 (Leica Microsystems Pty Ltd, Germany). Anther length was then measured directly from the image as a length of polygonal line segments drawn through the centre of the anther. Measurement was performed either manually by using image software FIJI (https://fiji.sc/) (Schindelin et al. 2012) or semi-automatically by using a Matlab (MathWorks, MA, USA) program written for this project (available on request). A minimum of at least five anthers collected for each spike sample was measured. A mean of five anthers from each spike represents AL value of each spike and means of AL values from three spikes were used for further statistical analysis. (4) Plant height (PH) was measured from the soil surface to the base of the flag leaf (mapping populations) or top of spike (NILs) at full maturity. (5) Spike length (SL) was recorded at full maturity and measured from the bottom of the spike to the top of the terminal spikelet excluding awns. (6) Spikelet number (SN) was counted at full maturity. (7) Awnedness (AW) was recorded as the presence or absence of awns. (8) Severe dwarf (SD) phenotype in population #1 was determined by visual assessment of plant stature at 8 weeks. It was also deemed when plant height at maturity was less than 50 cm or the plant contained AA alleles at chromosome 2B locus associated with SD or both. Since the SD phenotype greatly affected all the measured pollinator traits, F_2 plants with SD phenotype (51 plants) were excluded from data analysis of population #1 (see details in Supplementary document I).

DNA extraction, marker development and genotyping

DNA was extracted from leaf samples of parental lines and F_2 plants as previously described (Rogowsky et al. 1993). Genotyping of populations #1 and #2 was determined by genotyping-by-sequencing (GBS) markers and

supplemented by KASP™ markers using a subset of LGC Wheat KASP™ markers (https://www.lgcgroup.com/wheat/#.Wfvz22iCxaQ) and markers for *Rht-B1* and *Ppd-D1* [CerealsDB: (Wilkinson et al. 2012)] listed in Table S1. Population #3 and #4, additional population #2 and NILs were only genotyped by KASP™ markers for *Rht-1* and *Ppd-1*. KASP™ data were analysed by Kraken™ software (LGC Ltd, UK). GBS libraries for mapping population #1 and #2 were generated as described elsewhere (Elshire et al. 2011; Poland et al. 2012). DNA concentration was quantified by a standard PicoGreen (Thermo Fisher Scientific) assay and 200 ng of genomic DNA was digested by *Pst*I and *Msp*I restriction enzymes at 37 °C for 2 h, followed by ligation with 96 multiplex oligo adapters. Subsequently, all samples in the 96-well plate were pooled into a single 1.5 mL tube and pooled DNA was purified by ISOLATE II PCR and Gel Kit (Bioline, UK). The multiplex libraries were amplified by PCR and sequenced by Illumina HiSeq (Illumina Inc, USA) to obtain 150 bp paired-end sequences, according to the manufacturer's instruction. Genotypes for each individual were determined from the sequence data using an analysis pipeline as described elsewhere (Watson-Haigh and Eckermann, in preparation). Briefly, reads were aligned to the IWGSC RefSeq v1.0 genome assembly and the position of homozygous and polymorphic SNPs observed in the parent data was used to call SNPs in the F_2's. The GBS markers developed and used for linkage map construction of populations #1 and #2 are listed in Tables S2 and S3.

Linkage map construction, QTL analysis and statistics

Genetic linkage maps for populations #1 and #2 were generated by utilising the R package ASMap (Taylor and Butler 2017). Summary information for the linkage maps is shown in Table S4. Phenotypic data from the experiments in the first year (populations #1 and #2) were spatially analysed using ASReml (Gilmour et al. 2009) which was possible due to the replication of parental lines. For each trait, tray, row and column effects (Fig. S1) were fitted as random effects and lines fitted as fixed effects, which enabled best linear unbiased estimators (BLUEs) to be calculated for each F2 and parental line. QTL analysis was performed using the "scanone", "makeqtl", "addqtl", "refineqtl" and "fitqtl" from the R/QTL package (Broman et al. 2003), using all significant QTLs as covariates. A permutation test (using 1000 permutations) was used to set a LOD score threshold of 3.7 that corresponded to a genome-wide significance level of 0.05. Only QTLs above this threshold have been reported in this study in order to focus on higher effect QTL. Percent of phenotypic variation explained by QTL and the additive effect of the higher allele were also calculated. Summary statistical data for mapping populations, Pearson's correlation analysis, analysis of variance (ANOVA) for marker-trait association, and graphs (histogram, box plot, jitter plot and scatter plot) were produced by using GenStat ver15 (VSN_International 2011) and RStudio (RStudio_Team 2015).

Locating markers and genes/loci of interest on the Chinese Spring physical map

We mapped the following markers/genes/loci to IWGSC RefSeq v1.0, the Chinese Spring (CS) reference sequence assembly (International Wheat Genome Sequencing 2018); (1) GBS and KASP™ markers used in this study (Tables S1–S3), (2) markers corresponding to the QTL peak of pollinator traits identified in this study, (3) anther extrusion loci and their associated markers reported previously, where sequence information was available (Table S5) and (4) phenology genes/loci associated with flowering time and plant/floral architecture in rice, barley and wheat (Table S6). Marker and gene sequences were used for BLAST searches against the IWGSC RefSeq v1.0 and top hits with a significant similarity (100% identity or e value $< 1E-20$) to the expected chromosome, according to the literature, were used for mapping location on the CS physical map. Markers which showed multiple BLAST hits with similar e value to the same chromosome, they were not included in the map. The start sequence position (in Mb) of the BLAST alignment was used, and a physical map for each chromosome was generated using MapChart (Voorrips 2002).

Results

Development of genetic mapping populations and correlations of pollinator traits

Five spring wheat lines were used in this study to develop F_2 mapping populations, and they exhibited contrasting phenotype for a range of pollinator traits, especially AE and AL (Table 2). CIMMYT lines cid423295sid45 and cid388412sid46 were used as low AE parents, while Glenwari, H1621 and Gamenya were used as high AE parents. All mapping populations have allelic variation for *Rht-B1* and *Ppd-D1*, while all parental lines were monomorphic for *Rht-D1a* allele. Over 200 F_2 plants of populations #1 (cid423295sid45 × Glenwari) and #2 (cid388412sid46 × H1621) were phenotyped for plant height (PH), days to heading (DH), anther extrusion (AE), anther length (AL), spike length (SL) and spikelet number (SN). We observed a generally normal distribution for all six pollinator traits in population #1 (excluding SD plants; see Supplementary document I) and population #2 (Fig. S2). Since F_2 plants were used for phenotyping, we used replicated parental plants in order to adjust F_2 measurements for spatial

Table 2 Wheat accessions used for parents of F_2 mapping population, their traits and genotype

Accession name	Population[a]	Genotype			Trait[d]					
		Rht-B1[b]	*Rht-D1*[b]	*Ppd-D1*[c]	DH[e] (Day)	AE (scale)	AL (mm)	PH (cm)	SL (cm)	SN (No.)
cid423295sid45	Pop#1	Dw	Wt	INS	58.0	0.93	2.71	48.3	9.5	17.8
Glenwari	Pop#1, 4	Wt	Wt	SEN	67.4	3.92	3.98	67.5	11.6	20.1
cid388412sid46	Pop#2, 3, 4	Dw	Wt	INS	72.3	1.30	3.32	57.7	11.2	21.5
H1621	Pop#2	Wt	Wt	SEN	84.4	3.94	4.06	95.6	11.1	18.8
Gamenya	Pop#3	Wt	Wt	SEN	59.2	3.48	3.61	46.2	8.4	18.4

DH days to heading, *AE* anther extrusion, *AL* anther length, *PH* plant height, *SL* spike length, *SN* spikelet number

[a]Biparental F_2 genetic mapping populations developed with these lines as described in "Materials and methods" section

[b]*Rht-B1* and *Rht-D1* genotype determined by KASP markers. An allele for tall plant is "Wt", and semi-dwarf is "Dw"

[c]*Ppd-D1* genotype determined by KASP marker. An allele for photoperiod insensitive is "INS", and sensitive allele is "SEN"

[d]Trait data were obtained from plants grown in 2014 season, except for Gamenya, which was grown in 2015 season

[e]Value of measured traits. Adjusted means (BLUEs) were provided except for Gamenya

Table 3 Correlation between traits in mapping populations #1 and #2

	AE	AL	DH	PH	SL
Pop#1					
AL	0.15				
DH	−0.29***	0.32***			
PH	0.13	0.39***	0.32***		
SL	−0.14	0.58***	0.61***	0.33***	
SN	−0.18*	0.32***	0.79***	0.31***	0.74***
Pop#2					
AL	0.23***				
DH	−0.16*	−0.09			
PH	0.27***	0.18**	0.44***		
SL	−0.08	0.42***	0.12	0.02	
SN	−0.12	0.25***	0.46***	0.26***	0.59***

AE anther extrusion, *AL* anther length, *DH* days to heading, *PH* plant height, *SL* spike length, *SN* spikelet number

*, **, *** indicate significance of correlation $p<0.05$; $p<0.01$; $p<0.001$, respectively

and environmental effects derived from slight differences in growing conditions. Parental lines clearly exhibited large genetic variation for most traits relative to that deemed to be environmental variability based on parental replicates (Fig. S3). This indicated that the populations exhibited a significant genetic variance between F_2 individuals. We investigated correlations between pollinator traits, especially anther traits, in order to understand their functional relationship. In population #1, we found a moderate negative correlation between AE and DH and also that AL was moderately and positively correlated with DH, PH, SL and SN (Table 3). In population #2, AE was weakly and positively correlated with AL and PH, whereas a negative correlation between AE and DH was observed to be weakly significant. AL was moderately correlated with SL and weakly with PH and SN. Overall, correlations of AE with other traits are generally weak, while AL correlated relatively higher with other traits in these two populations. These findings indicate that the physiological mechanisms and genetic factors responsible for determining AL may have more commonality with those determining flowering time, stature and floral organ size.

QTL analysis in F_2 population #1 identified a major effect of *Ppd-D1* locus on multiple pollinator traits

F_2 populations #1 and #2 were genotyped by GBS and KASP™ markers using approximately a thousand markers for each population (Tables S1–S3). Genetic linkage analysis resulted in 21 linkage groups for both populations, corresponding to each of the wheat chromosomes, with total genetic map size of over 4000 cM for each population (Table S4). Linkage maps for D chromosome tended to have fewer markers, e.g. 4D and 5D, due to shorter chromosome size and less availability of polymorphism, compared to those of A and B chromosomes. QTL analysis was conducted for all six pollinator traits as well as awnedness (AW) and severe dwarf (SD) in population #1. A strong QTL for AW at the distal end of chromosome 5A (Fig. S4a and Table 4) was identified which was co-located with the well-known dominant *B1* locus for awn inhibition (Kosuge et al. 2008). This provides confidence that the approach for linkage map construction and QTL analysis in this paper is able to accurately detect true loci.

QTL analysis in population #1 identified a single AE QTL of medium effect on chromosome 1B with LOD score of 5.88, explaining 15.5% of the phenotypic variation, for which the high allele is derived from elite pollinator parent Glenwari (Fig. 1a and Table 4). Similarly, a single AL QTL of medium effect was identified on chromosome 2D, with *Ppd-D1* as the closest marker to the QTL peak and the Glenwari *Ppd-D1b* photoperiod-sensitive allele having a positive effect (Fig. 1a

Table 4 QTL summary for pollinator traits

QTL name	Trait[a]	Chr[b]	Position (cM)[c]	Marker[d]	LOD score[e]	% PV[f]	High allele[g]	Additive effect[h]
Population #1—cid423295sid45 (AA) × Glenwari (BB)								
qAE_P1.1	AE	1B	96.4	GP1_132	5.88	15.5	BB	1.22
qAL_P1.1	AL	2D	13.0	Ppd-D1	6.48	16.9	BB	0.29
qDH_P1.1	DH	2B	88.0	GP1_256	4.01	7.0	AA	4.87
qDH_P1.2	DH	2D	13.0	Ppd-D1	16.73	35.2	BB	12.93
qPH_P1.1	PH	4B	49.1	Rht-B1	18.91	41.8	BB	17.06
qSL_P1.1	SL	2D	13.0	Ppd-D1	14.32	31.7	BB	2.33
qSN_P1.1	SN	2B	86.0	GP1_256	3.90	8.5	AA	1.81
qSN_P1.2	SN	2D	13.0	Ppd-D1	7.68	17.8	BB	3.01
qAW_P1.1	AW	2B	156.3	GP1_283	4.41	3.7	BB	na
qAW_P1.2	AW	5A	164.0	GP1_681	44.03	68.9	AA	na
qAW_P1.3	AW	5B	4.0	GP1_688	4.67	3.9	BB	na
Population #2—cid388412sid46 (AA) × H1621 (BB)								
qAE_P2.1	AE	4B	0.0	Rht-B1	8.83	15.4	BB	1.22
qAE_P2.2	AE	6B	96.0	GP2_690	4.16	6.9	BB	0.72
qAL_P2.1	AL	2D	38.0	Ppd-D1	9.53	17.2	BB	0.22
qAL_P2.2	AL	4B	40.0	BS00012006	4.19	7.1	BB	0.17
qDH_P2.1	DH	2A	70.3	GP2_139	15.93	19.4	AA	8.72
qDH_P2.2	DH	2D	46.0	Ppd-D1	19.30	24.5	BB	8.46
qDH_P2.3	DH	5B	150.5	GP2_579	12.07	14.1	BB	6.51
qPH_P2.1	PH	2A	27.9	BS00023215	4.89	4.4	AA	5.56
qPH_P2.2	PH	2A	82.0	GP2_148	6.23	5.7	AA	7.61
qPH_P2.3	PH	4B	0.0	Rht-B1	22.37	24.7	BB	20.67
qPH_P2.4	PH	5B	122.2	GP2_574	5.30	4.8	BB	9.10
qSL_P2.1	SL	2D	41.7	Ppd-D1	7.60	12.9	BB	1.07
qSL_P2.2	SL	4B	6.0	GP2_463	5.63	9.4	AA	0.58
qSL_P2.3	SL	7A	200.7	GP2_812	4.65	7.7	AA	0.80
qSN_P2.1	SN	2D	41.7	Ppd-D1	11.38	19.6	BB	2.98
qSN_P2.2	SN	7A	164.3	GP2_795	6.61	10.8	AA	2.25

AE anther extrusion, *AL* anther length, *AW* awnedness, *DH* days to heading, *PH* plant height, *SL* spike length, *SN* spikelet number, *na* not available

[a]Traits used for QTL analysis

[b]Chromosome

[c]Peak QTL position in the genetic linkage map

[d]Genetic marker closest to the peak QTL position

[e]LOD score for QTL peak. LOD 3.7 was used as a threshold of significant QTL

[f]% phenotypic variation explained by QTL

[g]Allele responsible for higher trait value

[h]Additive effect of high allele against low allele

and Table 4). This *Ppd-D1/2D* QTL was also significantly associated with DH, SL, SN indicating a major effect on these traits. A single PH QTL with major effect was identified at the *Rht-B1* locus on chromosome 4BS, and no other PH QTLs were detected. In this population, we were unable to confirm a genetic association between *Rht-B1* and AE trait where the LOD score at the *Rht-B1* marker was 0.73. However, in the AL QTL analysis, the LOD score at the *Rht-B1* marker was 2.45 which was below the threshold but does indicate some evidence for an effect on AL (see also Fig. S5a). Therefore, in population #1, allelic variation at *Rht-B1* does not affect AE, but may have a small effect on AL.

Rht-B1 locus is associated with anther extrusion in F_2 population #2

In contrast to population #1, the *Rht-B1* locus on chromosome 4B was identified as a major QTL for AE in population #2, with LOD score of 8.83, explaining 15.4% phenotypic variation (Fig. 1b and Table 4). This strong AE QTL was

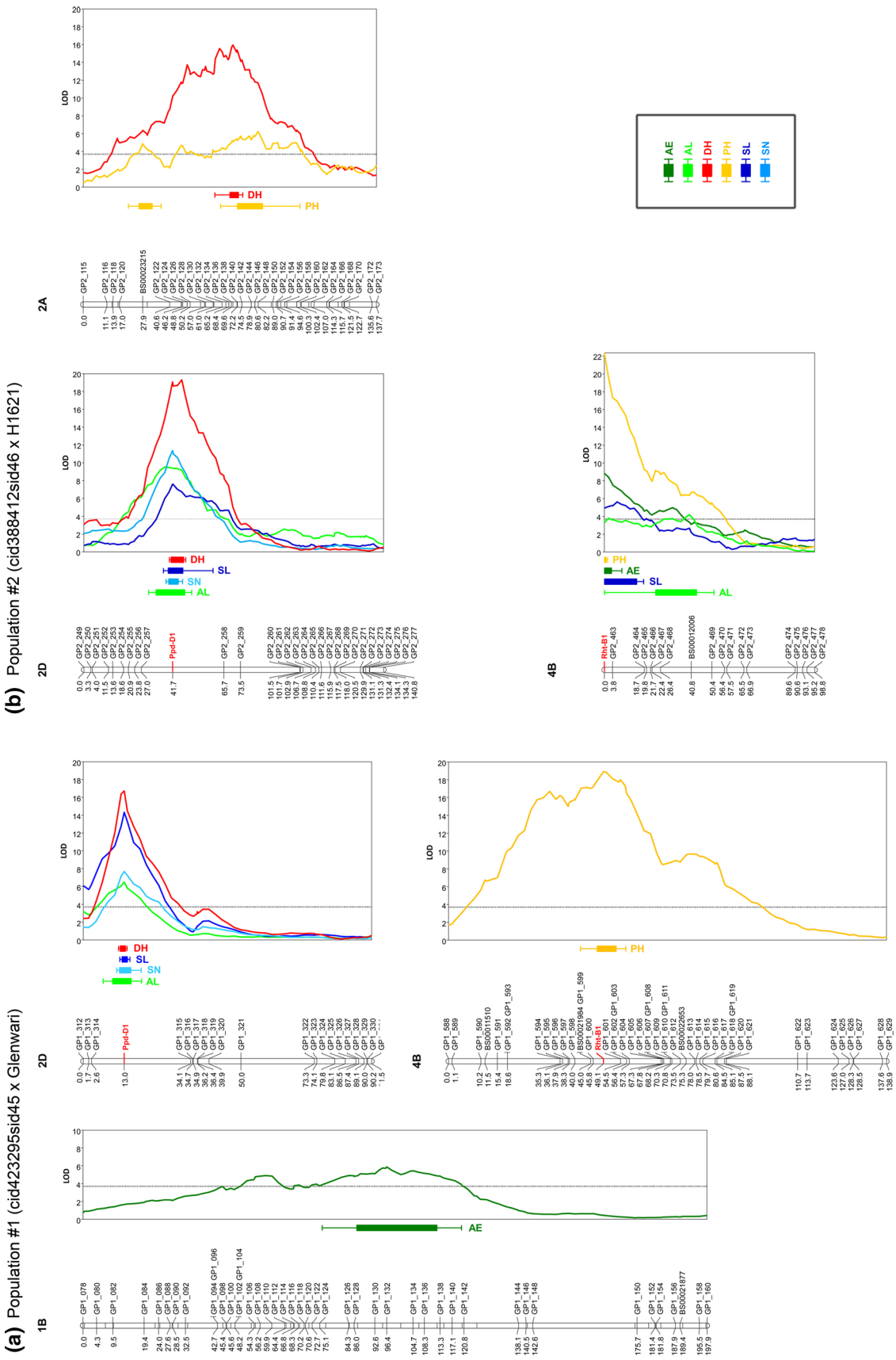

Fig. 1 Linkage maps and QTL positions for pollinator traits. Linkage map of each chromosome with genetic position (left) in cM scale and associated genetic marker name (right). Identified QTLs are presented at the right of each linkage map and shown in QTL graphs and bars. A threshold of LOD 3.7 is indicated by dashed line in the LOD graph with LOD score on the *y* axis. A QTL bar indicates LOD decrease of 1 (filled box) and decrease of 2 (outer border) from the maximum LOD value. Each trait is presented in different colour codes as indicated in the bottom right panel. **a** QTLs identified in population #1. **b** QTLs identified in population #2. Due to the high density of markers in the linkage map 1B and 2A, only even number GBS marker labels were shown. *AE* anther extrusion, *AL* anther length, *DH* days to heading, *PH* plant height, *SL* spike length, *SN* spikelet number

evident despite the 4B linkage map containing fewer markers and missing distal 4BS markers (Fig. 1b). An additional minor AE QTL was found on 6B spanning a broad region (148 cM, 95% confidence interval in Fig. S4b). This indicates a large uncertainty about the location of a QTL in this region, or possibly indicates the presence of multiple minor QTLs. As expected, the *Rht-B1/4B* locus was identified as a major QTL for PH and this QTL was also associated with SL. It should be noted that the *Rht-B1* locus corresponds to the second AL QTL peak on 4B, exhibiting a LOD score of 3.80 (Fig. 1b), while the first AL QTL peak was detected at 40 cM position on 4B. For these pollinator traits AE, AL and PH, the *Rht-B1a* tall wild-type allele derived from elite pollinator parent H1621 had a positive effect (Fig. S5a).

Similar to population #1, the *Ppd-D1/2D* locus showed a significant association with multiple traits including DH, SL, SN and AL with medium to high effects (Fig. 1b and Table 4). Again, the *Ppd-D1b* photoperiod-sensitive allele derived from the elite pollinator parent H1621 had a positive effect on all of these traits. We identified two additional QTLs for DH on 2A and 5B with medium–high effect and these QTLs were co-located with PH QTLs (Figs. 1b, S4b). However, none of these DH/PH QTLs showed associations with either AL or AE. Overall, *Rht-B1/4B* and *Ppd-D1/2D* loci have significant effects on multiple pollinator traits, with the tall *Rht-B1a* and photoperiod-sensitive *Ppd-D1b* alleles being associated with favourable pollinator traits.

Rht-B1 locus affects anther extrusion in a genotype dependent manner

We observed contrasting results for genetic association between the *Rht-B1* locus and trait AE in populations #1 and #2 (Fig. 1 and Table 4). This difference may be caused by allelic variation, not only of the *Rht-B1* gene itself but also other genes closely linked to the locus, and/or interaction between parental genetic backgrounds and epistatic interactions. Therefore, we subsequently investigated potential phenotypic variation in *Rht-B1* locus containing wild-type tall *Rht-B1a* allele between elite pollinator parents. We developed two additional F_2 populations by crossing the same low AE parent cid388412sid46 as population #2 and two different high AE parents, Gamenya for generating population #3 and Glenwari for population #4 (see "Materials and methods" section and Tables 1, 2).

The *Rht-B1* genotype was significantly associated with PH across all three populations (Fig. 2a), although additional dwarfing genes derived from Gamenya appeared to be present in population #3 as it has short stature (Table 2). We again found a significant association between *Rht-B1* genotype and AE in population #2, confirming our previous result (Figs. 2b, S5), but found no association in population #3 and only weak association

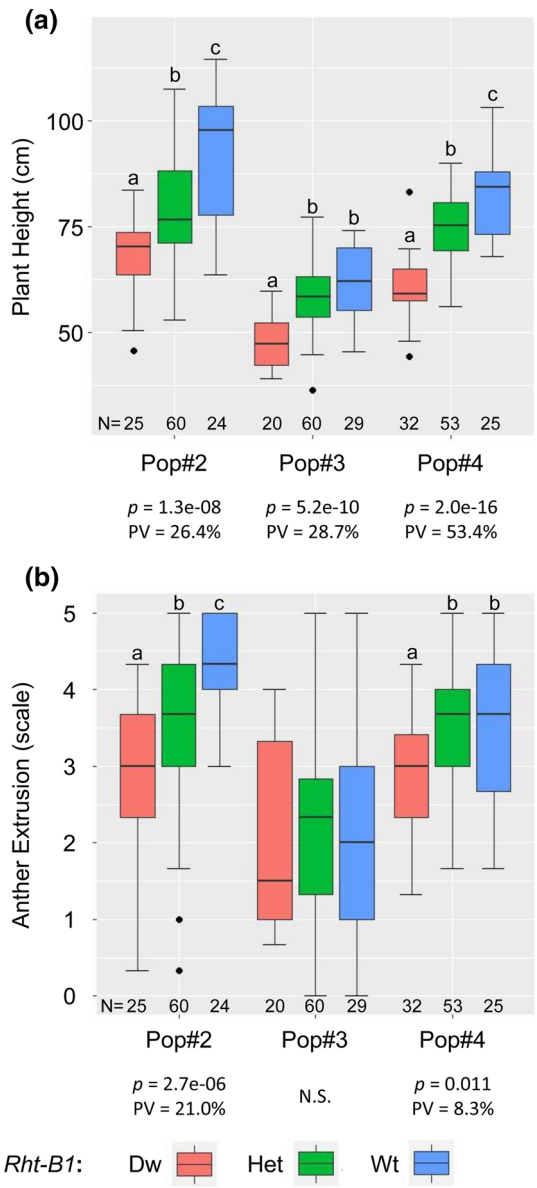

Fig. 2 Association of plant height (**a**) and anther extrusion (**b**) trait with *Rht-B1* genotype in three F_2 mapping populations (Pop#2, 3, and 4), presented by boxplot. *Rht-B1* genotype on *x* axis is indicated as follow: Dw; *Rht-B1b* homozygous semi-dwarf plant (red), Het; *Rht-B1a:B1b* heterozygous plant (green), Wt; *Rht-B1a* homozygous tall plant (blue). Association of *Rht-B1* genotype with PH or AE trait (*y* axis) was examined by ANOVA test and *p* value is presented below, if there is a significant association. N.S. indicates no significant association. Phenotypic variation explained by *Rht-B1* genotype is presented as PV%. Number of plants for each genotype group is indicated at the bottom, and groups with different index letters are significantly different based on a Tukey test at $p < 0.05$

in population #4. Furthermore, the same high AE parent Glenwari was used in population #1 and #4 and revealed slightly different effect of *Rht-B1* locus on AE trait for these two populations. These results suggest an epistatic

effect on AE between *Rht-B1* locus and the genetic background.

This was further investigated by using near isogenic lines (NILs) of the *Rht-B1* locus in two different recurrent parent cultivars (April Bearded and Mercia), minimising genetic background effect. Summary statistics for traits in NILs used in this study are shown in Table S7. NILs containing the *Rht-B1b* dwarf allele showed a significant reduction in PH as expected (Fig. 3). The *Rht-B1* locus also showed a significant association with AL in both NILs, which is consistent with AL QTL results for population #2 (Fig. 1). However, we found relatively larger trait variation in AE and no significant association with AE was identified in both NILs. These results further support our observation that an effect of *Rht-B1* allele on AE is genotype dependent, whereas there is more consistent association of *Rht-B1* locus with AL across genetic materials.

Rht-D1 and *Ppd-1* loci influence anther length

We also investigated the effect of *Rht-D1* and *Ppd-B1* and *Ppd-D1* loci on various pollination traits by using NILs. The *Rht-D1* NILs in all five recurrent parent backgrounds clearly showed a significant and consistent association with PH and AL (Fig. S6). All NILs containing semi-dwarfing *Rht-D1b* allele exhibited reduced PH and AL. The association with AE differed among recurrent parent genotypes, with three NILs (APD0, Huntsman and Nainari) showing a significant association with reduced AE, Mercia NIL having no significant association and April Bearded NIL having opposite association with increased AE. In contrast to results for the *Rht-B1* NILs, *Rht-D1* allelic variation showed no association with SL in three NILs (April Bearded, APD0 and Nainari). Indeed, an opposite effect was observed in two NILs (Huntsman and Mercia), indicating that the *Rht-D1b* semi-dwarfing allele is associated with longer spikes for these two genetic backgrounds (Figs. 3, S6).

Four types of *Ppd* NILs in spring wheat photoperiod-sensitive variety Haruhikari background (Tanio and Kato, 2007) were used in this experiment. NILs H(A) and H(B) carry the *Ppd-D1a* photoperiod-insensitive allele derived from Saitama27 and Fukuwasekomugi, respectively. H(C) NIL contains *Ppd-B1a* insensitive allele and H(D) carries *Ppd-B1a* and *Ppd-D1a* alleles derived from Fukuwasekomugi. As expected for the growing conditions, all NILs flowered significantly earlier than the recurrent parent line (Fig. S7). They also all exhibited significantly reduced AL except for the H(B) NIL. Similarly, both *Ppd-D1* and *Ppd-B1* NILs revealed a weak association with AE. Furthermore, *Ppd-D1* NILs [H(A) and H(B)] showed a strong association with SL. These results further confirmed the association of the *Ppd-D1* locus with multiple pollinator traits, especially AL and SL with the photoperiod-sensitive *Ppd-D1b* allele being associated with favourable pollinator traits (Figs. S5 and S7).

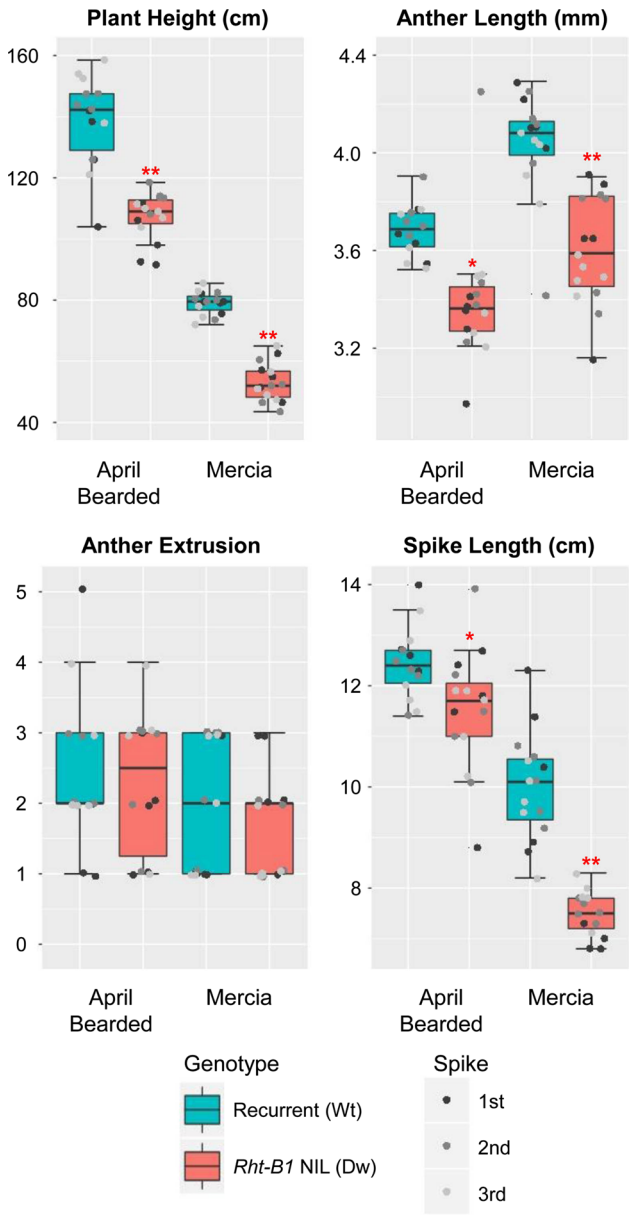

Fig. 3 Association of pollinator traits with *Rht-B1* allele in near isogenic lines (NILs). Recurrent parent cultivars, April Bearded and Mercia, have *Rht-B1a* homozygous allele (Wt) and corresponding NILs have *Rht-B1b* homozygous semi-dwarfing allele (Dw). Measurement data from first three spikes (in different dot colour) of five plants are presented by jitter plots overlays of box plots. A significant difference between recurrent parent and NIL examined by ANOVA is presented by asterisks: $*p < 0.01$; $**p < 0.001$

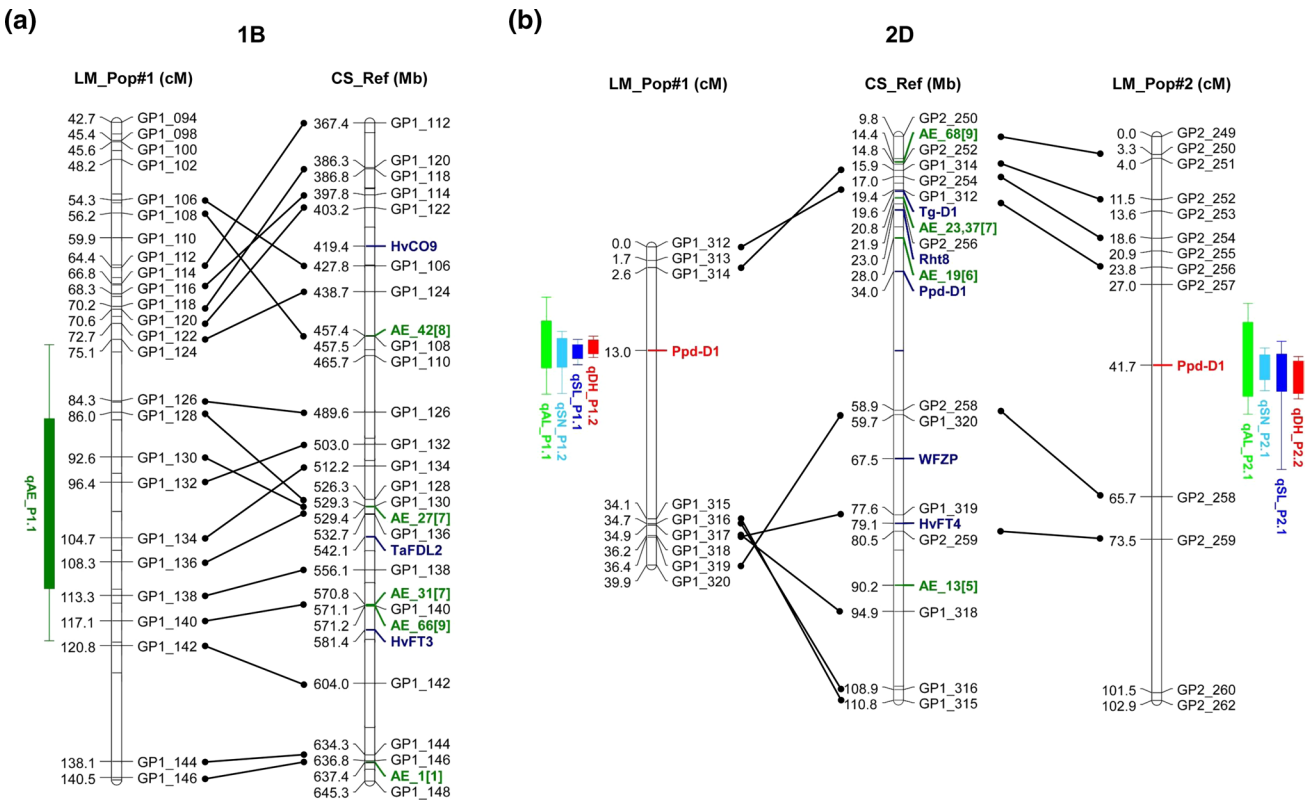

Fig. 4 A comparison between genetic map of this study and Chinese Spring reference sequence IWGSC RefSeq v1.0 physical map. **a** Chromosomes 1B around AE QTL. Genetic linkage map for population #1 (left) with associated pollinator trait QTL and markers were shown in cM scale. CS physical map (right) for corresponding chromosome region is presented by physical distance in Mb scale and linked markers were connected by lines. **b** Chromosomes 2D around *Ppd-D1* locus. Genetic linkage maps for population #1 (left) and population #2 (right) with associated pollinator trait QTLs and CS physical map (middle) for corresponding chromosome region are presented. Associated trait QTLs from this study (coloured bars, see Table 4) are shown beside genetic linkage map, while GBS markers (black letter, Tables S2–S3), phenology genes/markers (blue, Table S6) and previously reported anther extrusion loci/markers (green, Table S5) are drawn at the right side of both genetic and physical maps. Due to the high density of markers in the linkage map 1B, only even number GBS marker labels are shown in **a**

Physical location of anther extrusion loci and phenology genes in Chinese Spring reference genome map

AE or anther retention is reported to be controlled by multiple genetic factors of weak to medium effect in both spring- and winter-type wheat (Boeven et al. 2016; Muqaddasi et al. 2016, 2017a). However, all the reported genetic analyses for AE have been done by using different genetic mapping populations, including double haploid (Buerstmayr and Buerstmayr 2016; He et al. 2016a; Lu et al. 2013), recombinant inbred lines (Buerstmayr and Buerstmayr 2015; He et al. 2016a; Lu et al. 2013), backcross populations (Buerstmayr and Buerstmayr 2016) and association mapping populations (Boeven et al. 2016; Muqaddasi et al. 2016, 2017a; Würschum et al. 2018). It would be most valuable for breeding purposes if we could identify common AE QTLs in these studies. To achieve this, we mapped all the AE loci, including pollinator trait QTLs identified in our study, onto the Chinese Spring (CS) IWGSC RefSeq v1.0 physical map (International Wheat Genome Sequencing 2018). In addition, we mapped wheat phenology genes/loci associated with flowering time and plant/floral architecture to investigate physical distance between AE loci and phenology genes/loci.

GBS and KASP™ markers used in this study were also mapped to get an estimate of differences between genetic distance and physical distance in the CS reference genome. These markers were mapped to a greater extent in the distal chromosomal regions and less frequently in the central region, which indicates that centromeric or repetitive genomic regions act as marker poor domains (Fig. S8). A comparison of marker position and order in the genetic and CS physical maps shows differences between the two maps and where disagreements exist (Figs. 4, S9). The AE QTL we identified on chromosome 1B (qAE_P1.1) appears to be located near three other previously identified AE loci (Fig. 4a). About the broad AE QTL on 6B (qAE_P2.2)

identified in population #2, we found one known AE locus mapped relatively close to the QTL peak and several others located within broader QTL region (Fig. S9). Three known AE loci are physically closely located with *Ppd-D1* on chromosome 2D (Fig. 4b). There are a few other phenology genes/loci (e.g. *Rht8*, *WFZP* and *Tg-D1*) located nearby, which also could potentially affect the AE trait. Furthermore, there are several loci containing a few AE QTLs identified in common from independent genetic mapping projects, including regions on chromosome 2B near *Ppd-B1* and *Tg-B1*, 4B at *Rht-B1*, 4D at *Rht-D1* and 5B near *VRN-B1* (Fig. S8). It is reasonable to find that these AE loci are closely mapped to phenology genes, as such genes tend to have pleiotropic effect. Table S8 summarises physical position of AE loci and phenology genes that are located nearby (also see Fig. S8). These highlight the complexity of AE trait control as a result of contributions by multiple genetic loci and potential association with genes involved in flowering time and plant/floral architecture.

Discussion

Effect of *Rht-1* on anther extrusion

The use of elite pollinators is critical for hybrid wheat breeding programs. This requires multiple traits that have rather complex biological and physiological mechanisms and that are controlled by multiple genetic factors. Among these traits, AE is positively correlated with PH (Table 3 and Boeven et al. 2016; Langer et al. 2014; Lu et al. 2013) and associated with semi-dwarfing *Rht-1* loci (Boeven et al. 2016; Buerstmayr and Buerstmayr 2016; He et al. 2016b; Lu et al. 2013). In our study, we have shown that the effect of *Rht-B1* locus on AE is genotype dependent; only two out of four F2 populations investigated revealed a significant association (Figs. 1, 2 and Table 4). A few previous GWAS studies did not find an association of AE with *Rht-B1* locus, partly due to the small number of plants carrying *Rht-B1b* semi-dwarfing allele in the mapping population (Boeven et al. 2016; Muqaddasi et al. 2016, 2017a, b), and another study showed no significant association of *Rht-D1* locus with AE in a population of 131 RILs (He et al. 2016a). Combined with our results, *Rht-B1* appears to affect AE in a more genotype dependent manner, whereas *Rht-D1b* semi-dwarfing allele tended to show a stable negative effect. Nevertheless, high AE wheat accessions carrying semi-dwarfing alleles of *Rht-B1b* or *Rht-D1b* do exist (Würschum et al. 2018). Genotype dependency of *Rht-1* association with AE may be explained by the requirement of multiple biological processes for this trait. At anthesis, lodicule swelling induces flower opening followed by anther filament elongation and therefore anthers extrude. Shape of spikelet and floret, spikelet spacing, lodicule and anther size, filament length and glume stiffness are all likely to play a role in influencing the level of flower opening and anther extrusion process. Positive correlation between AE and AL (Table 3), AE and AL plus filament length (Langer et al. 2014) and co-localisation of glume stiffness and AE QTLs (He et al. 2016a) partly supports this idea. Some influencing factors listed above may affect AE more positively, overcoming negative effects of *Rht-1* semi-dwarfing alleles.

Rht-1 encodes a DELLA protein which plays a part in the gibberellin signalling pathway and is involved in various growth processes such as seed germination, stem elongation, leaf expansion, trichome development, pollen maturation, and the induction of flowering (Daviere and Achard 2016; Pearce et al. 2011). Therefore, mutation of this gene generally causes pleiotropic effects. Our genetic study revealed that *Rht-B1* locus is not only associated with AE and PH, but also AL and SL (Figs. 1, 3, S5 and Table 4). We also showed a significant association of *Rht-D1* with AL (Fig. S6). Thus, *Rht-1* is involved in multiple traits influencing AE process, including spikelet spacing (SL), anther size (AL), glume stiffness (Buerstmayr and Buerstmayr 2016), and filament elongation (Youssefian et al. 1992). This could explain why *Rht-1* is the most effective QTLs for AE.

Tall male pollinators with high AE associated with *Rht-B1a* or *Rht-D1a* wild-type alleles are desirable for hybrid seed production. However, F1 hybrids would inherit tall alleles, which is likely to be highly undesirable in some environments. Semi-dwarf wheats yield more and are typically selected for hot and dry climates (Alghabari et al. 2014; Kowalski et al. 2016; Tricker et al. 2018). It might be possible to use other *Rht* genes for introducing semi-dwarf trait. The *Rht24* locus, for example, did not show a significant effect on AE (Würschum et al. 2018). We also confirmed that there is no known AE QTLs nearby *Rht24* on chromosome 6A (Fig. S8). Therefore, *Rht24* could be used to select semi-dwarf wheat plants without affecting AE trait (Würschum et al. 2018). Other *Rht* loci (*Rht12*/5A, *Rht23*/5D, *Rht13*/7B) did not co-locate with any known AE loci on the CS physical map, providing potential alternative semi-dwarfing genetic resources. In specific genetic backgrounds, it might also be possible to use semi-dwarfing allele *Rht-B1b* for controlling plant height without compromising AE (Figs. 2, 3). Better understanding of the effect of different *Rht* genes on AE and other pollinator traits could provide us with a greater choice of dwarf genetic resources to design superior hybrids in breeding programs.

Pleiotropic effect of *Ppd-D1* locus on pollinator traits

Optimal co-occurrence of flowering time between the male and female plants is one of the most important key factors in ensuring successful hybrid seed production. We have

shown that *Ppd-D1* locus not only affects flowering time (DH), but is also associated with important pollinator traits SL, SN and AL (Figs. 1, 4b). Anther size represented by AL is strongly correlated with pollen mass (Milohnic and Jost 1970; Nguyen et al. 2015; Pickett 1993). A long spike containing many well-spaced spikelets would facilitate flower opening as well as pollen dispersal, therefore favourable pollinator traits. Moreover, we found that *Ppd-D1* is weakly but significantly associated with AE in population #1 and NILs (Figs. S5b and S7), and three AE QTLs closely located with *Ppd-D1* (Fig. 4b) were identified in other studies (Boeven et al. 2016; Muqaddasi et al. 2016, 2017b). Similarly, *Ppd-B1* showed a significant association with AE and AL (Fig. S7) and two AE QTLs (Boeven et al. 2016; Muqaddasi et al. 2017a) mapped nearby *Ppd-B1* linked markers on chromosome 2B (Fig. S8). These demonstrated a pleiotropic effect of *Ppd-1* loci on multiple pollinator traits.

Ppd-1 encodes a pseudo-response regulator involved in the regulation of *CONSTANS* (*CO*) gene expression and upregulation of *VRN3*/*TaFT*, accelerating flowering under long days in wheat and barley (Distelfeld et al. 2009; Turner et al. 2005). In wheat, the semi-dominant photoperiod-insensitive *Ppd-D1a* allele has a deletion in the promoter that causes miss-expression of this gene and increased expression of *VRN3*/*TaFT* under short days, therefore inducing early flowering (Beales et al. 2007). Investigation of six haplotype variations of *Ppd-D1* revealed a significant haplotype effect on various agronomic traits, not only DH but also SL, SN and PH (Guo et al. 2010). It would be interesting to explore the effect of different *Ppd-D1* haplotypes on AL and AE traits as we found in H(A) and H(B) NILs (Fig. S7). Another allelic variant of *Ppd-D1* caused a change in spike architecture, forming paired spikelets (Boden et al. 2015), which could also influence pollinator ability. Therefore, *Ppd-D1* has a major effect on multiple pollinator traits and specific effects of haplotype variants on these traits needs to be investigated further.

Elite pollinators for hybrid wheat breeding

Mapping of known AE loci on wheat genome sequence (Fig. S8) highlighted the presence of multiple genetic factors associated with this trait, with each locus having a relatively minor effect (Muqaddasi et al. 2017a; Würschum et al. 2018). A comparison between our genetic linkage maps and CS physical map revealed a close link between AE QTLs (1B and 6B) and other flowering time genes/loci (Figs. 4a and S9). *TaFDL2* (Abe et al. 2005; Li and Dubcovsky 2008) and *HvFT3* (Halliwell et al. 2016; Mulki et al. 2018) are closely located to 1B QTL, while several *CO* genes (Griffiths et al. 2003; Nemoto et al. 2003) and *TaTOC1* (Zhao et al. 2016) are located within the broad 6B QTL. These genes are not only involved in flowering time, but many are known to have a pleiotropic effect on floral development; therefore, they may be affecting the AE trait. Nevertheless, these phenology genes, including *Rht-1* and *Ppd-1*, often affect flowering time as well as major agronomic traits (Guo et al. 2010; Richards 1992; Wilhelm et al. 2013; Youssefian et al. 1992). Since these are critical traits not only for hybrid seed production but also for general inbred line breeding, breeders may have limited scope for genotype selection beyond key phenology genes/loci already used in their current breeding program. Identifying new AE QTL loci with a significant effect within wheat genetic resources is one strategy, while the introgression of chromosome segments from related cross-pollinating grass species like rye could be another strategy to manipulate floral structure of wheat (Nguyen et al. 2015).

A more practical approach would rely on genomic selection for pyramiding multiple AE loci and other pollinator traits carrying favourable alleles and for predicting pollinator traits, flowering time and hybrid performance at the same time (Boeven et al. 2016; Okada and Whitford 2019). It is important to note that AE is a critical trait for hybrid seed set (Boeven et al. 2018), but not a sole trait determining elite pollinators. Many spikelets containing large anthers increase the total amount of pollen available for dispersal and these traits are under independent genetic control. Furthermore, female parent traits such as flower opening (Okada et al. 2018), stigma receptivity and length (Pickett 1993) are also critical and the genetic basis of these traits is still largely unknown in wheat. Prioritising pollinator traits in breeding selection programs is essential, and this will be facilitated by quantitative assessment of each pollinator trait and its impact on cross pollination efficiency. With recent advancements in phenotyping platforms (Ghanem et al. 2015; Gils et al. 2013; Jimenez-Berni et al. 2018), and the availability of wheat genomic resources (International Wheat Genome Sequencing 2018) and the implementation of new genetic modelling tools (Miedaner et al. 2017; Zhao et al. 2015), there is a real potential to breed elite pollinator lines, pyramiding multiple favourable traits and achieving superior performance in F_1 hybrids at the same time.

Author contribution statement TO, RJ, UB, MA, PW and RW planned and designed the research. TO, RJ, PE, NW, PW, YH, MB, ET and HL performed experiments. TO, RJ, PE, NW, YH, MB and ET analysed data. TO, RW, RJ, MA, PW, KK, MA, YH, DF and UB discussed results and interpretation of data. TO wrote the manuscript, and TO, PW, NW, PE, UB, RW edited the manuscript. All authors reviewed the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The authors declare that this study complies with the current laws of the countries in which the experiments were performed.

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