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Plant Science, 2015; 233:143-154

Originally published at:
http://doi.org/10.1016/j.plantsci.2015.01.008

Plant Science 233 (2015) 143–154

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Detection of QTL for metabolic and agronomic traits in wheat with adjustments for variation at genetic loci that affect plant phenology

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ARTICLE INFO

Article history:
Received 20 August 2014
Received in revised form 15 January 2015
Accepted 18 January 2015
Available online 29 January 2015

Keywords:
Drought
Metabolite
Phenology
Photoperiod
QTL
Wheat

ABSTRACT

Mapping of quantitative trait loci associated with levels of individual metabolites (mQTL) was combined with the mapping of agronomic traits to investigate the genetic basis of variation and co-variation in metabolites, agronomic traits, and plant phenology in a field-grown bread wheat population. Metabolome analysis was performed using liquid chromatography–mass spectrometry resulting in identification of mainly polar compounds, including secondary metabolites. A total of 558 metabolic features were obtained from the flag leaves of 179 doubled haploid lines, of which 197 features were putatively identified, mostly as alkaloids, flavonoids and phenylpropanoids. Coordinated genetic control was observed for several groups of metabolites, such as organic acids influenced by two loci on chromosome 7A. Five major phenology-related loci, which were introduced as cofactors in the analyses, differed in their impact upon metabolic and agronomic traits with *QZad-aww-7A* having more impact on the expression of both metabolite and agronomic QTL than *Ppd-B1*, *Vrn-A1*, *Eps*, and *QZad-aww-7D*. This QTL study validates the utility of combining agronomic and metabolomic traits as an approach to identify potential trait enhancement targets for breeding selection and reinforces previous results that demonstrate the importance of including plant phenology in the assessment of useful traits in this wheat mapping population.

1. Introduction

Drought is one of the major abiotic stresses that limit plant growth and productivity of crop plants, including bread wheat (*Triticum aestivum* L.) [1,2]. A plant's capacity to tolerate drought stress varies depending upon the developmental stage at which the stress is imposed [3]. It is essential for plants to adjust their life cycle to flower and mature when they are least at risk of exposure to abiotic stresses and therefore potential harm by adverse environmental conditions. Flowering time in wheat is genetically controlled and affected by vernalization (*Vrn*), photoperiod (*Ppd*), and development rate or "earliness per se" (*Eps*) genes [4,5]. *Vrn* and *Ppd* genes affect the plant's sensitivity to cold temperature and day length, whereas *Eps* genes influence flowering time by affecting the number and rate of initiation of vegetative and floral primordia irrespective of environmental factors. Recent studies suggest that plant metabolism can be modified through either classical breeding [6,7] or metabolic engineering [8–10], but the extent to which plant phenology influences metabolism or *vice versa* is currently not well understood.

Because of the diversity of structural classes of metabolites there is no single methodology that can measure the complete metabolome. Primary metabolites including carbohydrates, amino acids, and organic acids are easily measured by gas chromatography–mass spectrometry (GC–MS), but complex secondary metabolites, such as phenolics, alkaloids and terpenoids, will only be detected by using complementary techniques such as liquid chromatography–mass spectrometry (LC–MS) and nuclear

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http://dx.doi.org/10.1016/j.plantsci.2015.01.008

magnetic resonance (NMR). Using a combination of different instrument platforms and techniques can improve opportunities to reveal differences in the metabolome [11].

GC–MS-based methodologies offer a robust and well-established analysis workflow, but are restricted to low molecular weight metabolites which are either volatile or can be transformed into volatile and thermally stable compounds through chemical derivatization prior to analysis [12]. This includes especially primary metabolites, such as amino acids, amines, sugars, and organic acids. By contrast, LC coupled to hybrid quadrupole time-of-flight mass spectrometers (LC–QTOF-MS) is considered to be amongst the most versatile metabolite profiling technique [13], providing a tool to analyze a broad range of metabolites including secondary metabolites such as alkaloids, benzoids, flavonoids, terpenes, isoprenes and phenylpropanoids, and highly polar and/or higher molecular weight molecules, such as oligosaccharides and lipids. LC–MS typically uses ESI and produces either protonated (ESI^+; positive electrospray ionization mode) or deprotonated (ESI^-; negative electrospray ionization mode) molecular ions [14]. It allows for accurate mass measurement, isotopic pattern recognition and high sensitivity making it suitable for calculations of elemental composition of mass signals [15].

In a previous study [16] we employed GC–MS profiling to analyze predominantly primary metabolites of flag leaves of 233 doubled haploid lines of bread wheat (*Triticum aestivum* L.) from a cross between a drought tolerant (Excalibur) and a drought sensitive (Kukri) cultivar grown under terminal drought stress conditions. We build on this study and now analyze flag leaves of a subset of the population (179 lines) taken from the same experiment using LC–MS. Since elucidation of the relationship between plant phenology, agronomic and metabolic traits could support breeding efforts to improve abiotic stress tolerance and other agronomically important traits, the aims of this study were to (i) analyze the effects of five phenology-related loci on metabolic and agronomic data, (ii) estimate the genetic correlation coefficients between the agronomic traits, secondary metabolites, and agronomic traits and secondary metabolites after the data were adjusted genetically for the effects of the phenology-related loci, and (iii) estimate the locations and the effect sizes of metabolic QTL and agronomic QTL not associated with plant phenology on an improved Excalibur/Kukri genetic marker map.

2. Materials and methods

2.1. Bread wheat cultivars and mapping population

The plant population consisted of 233 doubled haploid lines produced from a cross between 'Excalibur' (RAC177/'Monoculm'//RAC311S; released by the University of Adelaide in 1991) and 'Kukri' (76ECN44/76ECN36//RAC549; MADDEN/6*RAC177; released by the University of Adelaide in 1999) [16].

2.2. Field experimental conditions

The field trials and the evaluation of agronomic traits were conducted in 2006 at the Roseworthy Campus at the University of Adelaide as described by Hill et al. [16]. The field experiment was randomized using a nearest-neighbor design with two replicates of each doubled haploid line, with additional plots of the parental lines and control varieties. The sampling of flag leaves was performed between 10:00 and 15:00 on 1 day, when most lines had reached anthesis. Samples were immediately stored at −80 °C until extracted.

2.3. Chemicals for metabolite profiling

All chemicals and solvents were purchased from Sigma–Aldrich (Australia) and were either of analytical or mass spectrometric grades. Deionized water (18.2 MΩ) was produced using a Synergy UV Millipore System (Millipore).

2.4. Sample extraction and preparation procedure

The samples were extracted and prepared using a modified version of the extraction protocol as described by Hill et al. [16,17]. Briefly, 0.5 mL 100% methanol was added to 30 mg of flag leaf tissue for each line. Homogenization was performed using cryo mill tubes (Precellys lysing kit) and a cryo mill (Precellys 24 lysis and homogenization unit), both purchased from Bertin Technologies, France. After addition of 20 μL internal standard solution (20 μL per sample from a stock solution containing 0.2 mg mL^{-1} ^{13}C sorbitol, ^{13}C valine, 2-aminoanthracene, and 2,3,4,5,6-pentafluorobenzoic acid, respectively, in 100% methanol), samples were extracted for 15 min at 70 °C, mixed vigorously with one volume of water, and then centrifuged for 10 min at 13,000 rpm. Aliquots (100 μL) of the supernatant were transferred into glass vial inserts and dried *in vacuo* for LC–MS analysis. The sample was resuspended in 50% methanol/water solution (100 μL) before injection of 2 μL onto the LC column. Pooled reference samples were injected every ten injections for quality control of the chromatography.

2.5. Liquid chromatography conditions

Separations were carried out using an Agilent Technologies (Santa Clara, CA, USA) 1200 LC system equipped with a Zorbax Eclipse XDB-C18 Rapid Resolution HT 2.1 × 50 mm, 1.8 μm column at a flow rate of 400 μL min^{-1}, maintained at 40 °C, resulting in operating pressures below 400 bar. Chromatographic separation was performed over a 16 min run time using an aqueous mobile phase (0.1% (v/v) formic acid in deionized water) as solvent A and an organic phase (0.1% (v/v) formic acid in acetonitrile) as solvent B following a linear gradient, increasing solvent B from 5% to 100%. The gradient was 5:95 (B:A) to 100:0 in 10 min, and then followed by a 2 min hold at 100:0 (B:A). A 4 min re-equilibration period at 55:95 (B:A) was established between injections.

2.6. Mass spectrometry conditions

The LC system was coupled to an Agilent Technologies 6520 ESI-QTOF-MS (Santa Clara, CA, USA) equipped with an ESI source for profiling and accurate mass experiments. The following ESI source conditions were used: sheath gas temperature 300 °C, gas flow rate 10 L min^{-1}, nebulizer pressure 45 psi, fragmentor 150 V, skimmer 65 V, and capillary voltage (4000 V for positive mode and 3500 V for negative mode).

MS/MS spectra were collected and monitored within the mass range 70–1700 m/z for both positive and negative ESI, using an acquisition rate of 3 MS spectra/second with a medium isolation width of 4 m/z amu and fixed collision energies of 10, 20, and 40 V. Scheduled precursor lists for targeted MS/MS experiments were created in the MassHunter (Agilent Technologies, Santa Clara, USA) data acquisition software.

Untargeted LC–MS data were acquired in a centroid data format. For each sample, the number of detected masses was reduced by exploratory analysis of the data set. Only masses that were detected in the optimized gradient phase (between 0.6 and 15 min retention time), with a signal intensity higher than six times local noise, and were detected in more than 50% of all samples were selected

for further data analysis. The final data set contained 469 (positive ionization mode) and 89 (negative ionization mode) mass features.

2.7. Targeted analysis

Targeted screening was carried out using precise extracted ion chromatograms (EICs), retention time and isotope pattern evaluation using MassHunter Workstation Software (Agilent Technologies, Santa Clara, USA) version B 05.00. An in-house accurate mass/retention time library (Metabolomics Australia) covering ~300 primary and secondary metabolites representing more than 20 compound classes including retention time, sum formula information, accurate mass, and MS/MS spectra for three collision energies (10, 20, and 40 eV) in both positive and negative ionization modes was used to identify the known compounds. Additionally, the Kyoto Encyclopedia of Genes and Genomes (KEGG; www.genome.jp/kegg) database was used in the Agilent MassHunter software to putatively identify compounds. Common adduct information was additionally taken into account to increase the confidence in compound identification by targeted analysis. Compounds which were putatively identified, but had an overall score of less than 75, were denoted as "NA" (not assigned) in this study. Since several mass features can correspond to a single compound some annotations remain assigned as "putative" as unequivocal identifications would entail a separate project for each identified compound, falling outside the scope of this study.

2.8. Normalization

All samples (two biological and two technical replicates) from all lines were completely randomized to minimize systematic variation in the data. Metabolite data were normalized by dividing the intensity of the ion by both the intensity of the internal standard (^{13}C sorbitol for positive ionization mode, and 2,3,4,5,6-pentafluorobenzoic acid for negative ionization mode), and the sample dry weight (g) as described by Hill et al. [18].

2.9. Linkage map

The molecular linkage map of the Excalibur/Kukri cross was originally constructed at the Australian Centre for Plant Functional Genomics (ACPFG) as described by Edwards [19]. The genetic markers used in this study consisted of 551 polymorphic markers including DArT (Diversity Arrays Technology), SSR (simple sequence repeats), and GBS (genotyping-by-sequencing) markers, genotyped for 190 lines of the E/K doubled haploid population. The final constructed linkage map consisted of 23 linkage groups with 1A and 7D split into two separate linkage groups that were considered to be the short and long arms of their respective chromosome. Missing marker scores were numerically imputed using the rules of Martinez and Curnow [20]. Interval midpoint pseudomarkers were calculated using the calculations derived in Verbyla [21]. Genetic distances were based on the Kosambi mapping function and estimated using the Hidden Markov algorithm of Lander and Green [22] implemented in the R/qtl package (http://CRAN.R-project.org/package=qtl). The linkage map had a total length of 3124 cM (centiMorgan; 1172.7, 1129.6, and 821.4 cM for the A, B and D genomes, respectively) and an average distance of 5.7 cM between markers.

2.10. Loci that affect phenology

Five specific genomic positions (one on each of chromosomes 2B, 4A, 5A, 7A and 7D) were selected to represent loci that Edwards [19] had mapped as affecting flowering time in the Excalibur/Kukri population. Two of these were represented by markers that detect sequence polymorphisms in the known phenology genes *Ppd-B1* (on chromosome 2B) and *Vrn-A1* (on chromosome 5A), one by a marker (*wmc0603* on chromosome 4A) at which Edwards [19] detected an earliness *per se* QTL, and two by inferred markers on chromosomes 7A and 7D. Estimation of the positions of the QTL *QZad-aww-7A* and *QZad-aww-7D* was undertaken using whole-genome analysis of Zadoks scores obtained from the field experiment, with the flanking marker methods described in Whittaker [23] used to infer marker genotypes at 0.1-cM intervals on chromosomes 7A and 7D.

2.11. Exploratory analysis and transformation

An initial exploratory analysis showed nearly all agronomic traits were normally distributed (data not shown). The traits grain size G2.8 and spikes per m^2 (SpM2) were the exception: these required log and square root transformation, respectively, prior to formal analysis. In contrast, the distributions of the metabolite traits were heavily skewed to the right (data not shown), and thus were log transformed prior to formal analysis. After transformation initial linear mixed models were fitted using the details of Section 2.12 and the models were diagnostically assessed to determine the feasibility of retaining each of the metabolites for the complete analysis. This assessment was based on a visual examination of model residuals and their requirement to satisfy model assumptions. Metabolites retained after this process were then checked for extreme outliers. If an observation exceeded four times the standard deviation and was also deemed to be a residual outlier from an initial model fit, it was set to missing for subsequent analyses.

2.12. Linear mixed model analysis

The analysis of the agronomic and LC–MS metabolite traits used the linear mixed model described by Hill et al. [16]. The model consists of a set of fixed and random effects that accounts for genetic and non-genetic sources of variation. Specifically, the non-genetic or extraneous sources of variation, due to the field or laboratory design, were captured using separate random effects. The model also captured the genetic variation by including a random factor with a level for all genotypes in the experiment. To ensure this variation was due to the doubled haploid lines only, the parental lines were fixed by incorporating a fixed factor comprising of a level for all the doubled haploid lines, and a separate level for each of the parents. For all agronomic and metabolic traits, the five phenology-related loci (Table 1) were fitted as additional set of additive fixed effects. This ensured that the doubled haploid genetic component of each trait was appropriately adjusted for loci known to affect plant phenology. As a consequence, the remaining genetic variation of the doubled haploid lines is a residual representation of the doubled haploid genetic component of the trait. For each of the traits, the best linear unbiased predictors (BLUPs) of the doubled haploid lines were extracted from the fitted model and the generalized heritability was calculated using the formula developed by Cullis et al. [24], namely

$$h_g^2 = 1 - \frac{PEF}{2\sigma_g^2}$$

where *PEV* is the average pairwise prediction error variance of the BLUPs and σ_g^2 is the genetic variance of the doubled haploid lines. The linear mixed modelling software ASReml-R (http://www.vsni.co.uk/software/asreml) available in the open source statistical software platform R (http://www.R-project.org) was used for analysis of all agronomic and metabolite models.

Table 1
Position of loci that affect plant phenology on the Excalibur × Kukri genetic map.

Locus	Full name	Reference	Position on Excalibur × Kukri map	
			Chromosome	Position (cM)
Ppd-B1	*Photoperiod insensitive*	[32,33]	2B	83.92
Vrn-A1	*Vernalization*	[31,69,70]	5A	111.75
Eps	*Earliness per se*	[35,68]	4A	86.48
QZad-aww-7A	–	[71]	7A	90.5
QZad-aww-7D	–	Unpublished	7D	0.53

2.13. Effects of phenology-related loci

From each of the agronomic and metabolic trait fitted models, the estimated effects of the phenology-related loci listed in Table 1 were extracted. The significance of each effect was then examined individually by forming a Wald statistic [25], namely

$$\frac{\hat{\theta}}{var(\hat{\theta})} \sim X_1^2 \quad (1)$$

where $\hat{\theta}$ is the estimated effect of the phenology-related locus. This statistic can be viewed as the square of the usual z-statistic and is known to have a chi-squared distribution with one degree of freedom. For an alpha level of 0.05, Wald statistics greater than 3.84 are considered to be significant.

2.14. Genetic correlations

For all metabolite traits, the BLUPs for the doubled haploid lines were extracted from each fitted model and the complete set of Pearson pair-wise genetic correlations were calculated. Similarly, pairwise genetic correlations between the agronomic and metabolite traits were calculated using BLUPs for the 177 doubled haploid lines that were included in both the field and laboratory experiments.

The significance of the metabolite genetic correlations was assessed using permutation. A total of 5000 permutations were performed with each permutation involving the random re-ordering of individual metabolite doubled haploid BLUPs and recalculation of all pairwise genetic correlations. Due to the large number of simultaneous tests being performed, the family wise Type I error required adjustment. This was achieved by considering only the absolute maximum correlation value for each permutation [26] and, over all 5000 permutations, calculating the empirical distribution of the maximum test statistic

$$t_{\max} = \frac{r_{\max}}{\sqrt{(1 - r_{\max})^2 (N - 2)}}$$

The empirically corrected P-value was then determined from the 95th percentile of this distribution and was calculated to be 1.70×10^{-7}. Adjusted P-values for individual correlations were then calculated by determining the number of empirical maximum correlations that exceeded the individual absolute correlations and dividing by the number of permutations conducted.

The discussion of the genetic correlations is based on the magnitude of the estimates as follows: (1) a correlation estimate greater than 0.5 was considered strong; (2) a correlation estimate between 0.3 and 0.5 was considered moderate; and (3) a correlation estimate less than 0.3 was considered weak.

2.15. QTL analysis

The whole genome average interval mapping (WGAIM) approach of Verbyla et al. [21,27] was used for QTL analysis of the agronomic and metabolic traits in this study as described Hill et al. [16]. The approach uses the linear mixed model for each of the traits ensuring the phenology loci were incorporated as cofactors during the analysis. The significance of each of the QTL was determined from the appropriate Wald statistic given in (1) where the QTL effect size is $\hat{\theta}$. The approach has been computationally implemented in version 1.4 of the R package wgaim (http://cran.r-project.org/web/packages/wgaim/index.html) [28] and uses ASReml-R for analysis of all models.

3. Results

3.1. Metabolic trait variation and comparison of different ionization modes

The mapping populations consisted of 233 Excalibur × Kukri doubled haploid lines which were grown in the field under terminal drought conditions. For a subset of 179 experimental lines, metabolite extractions were prepared from flag leaves obtained from two independent plants per line and analyzed in duplicate. Since many compounds may preferentially ionize in either ESI^+ or ESI^- using LC–MS, we analyzed extracts consecutively in both ionization modes and compared the absolute mass signal intensities, expressed in peak areas, of the parent ions of identified and unidentified mass features. A total of 558 compounds were detected (469 in ESI^+, and 89 in ESI^-), of which 197 (157 in ESI^+, and 40 in ESI^-) could be putatively identified using both our in-house library and the publicly available KEGG library. The putative identities of the detected compounds are listed in Supplemental Tables S1 and S2. All compounds were included in the analysis to investigate the degree of association with identified metabolites.

After putative annotation, compounds were grouped into 18 distinct classes (Table 2). The largest classes of identified compounds were alkaloids (26), flavonoids and derivatives (24), terpenoids (19) and organic acids (16). Nitrogen-containing compounds, such as alkaloids and amino acids as well as phenols, terpenoids, and fatty acids were among the putatively annotated compounds that ionized more efficiently in positive ionization mode, while many hydroxide-containing compounds, such as many organic acids and carbohydrates ionized more efficiently in negative ionization mode.

3.2. Effect of phenology-related loci on the metabolite levels and agronomic traits

The influence of five phenology-related loci on each of the metabolite levels and agronomic trait scores was estimated using a linear mixed model. This approach allowed the appropriate partitioning of genetic and non-genetic variation prior to correlation and QTL analysis. As the phenology loci were fitted as fixed effects, a Wald test [25] was used to gain a better understanding of the size and statistical significance of their effects on each of the traits. The Wald statistics (w) and corresponding P-values for the five loci across the complete set of metabolite traits are listed in Supplemental Tables S3 and S4, and are plotted in Fig. 1.

The five phenology-related loci were found to differ with respect to the number and identity of metabolites for which they had significant ($P < 0.05$) effects. *Ppd-B1* and *Vrn-A1* had a similar number

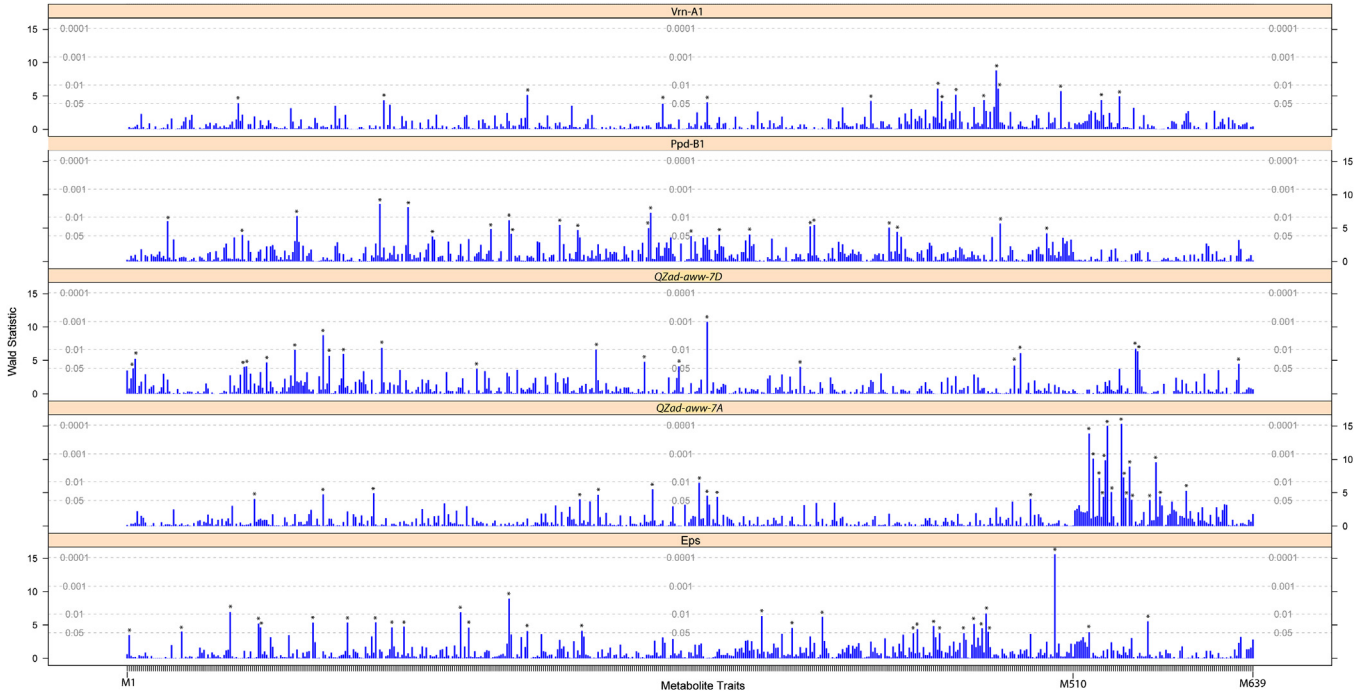

Fig. 1. Wald test statistic for the effect of each of the five maturity-related loci on the metabolic traits. Horizontal lines indicate different significance thresholds of the Wald statistic. Significant effects ($P < 0.05$) are marked with an asterisk. M1–M510: metabolites measured in ESI$^+$ mode; M511–M639: metabolites measured in ESI$^-$ mode. A fully annotated figure is provided as Supplemental Fig. S1.

of metabolites with significant Wald statistics (20 and 14 metabolites, respectively). The *Eps* locus on chromosome 4A affected more metabolites than *Ppd-B1* and *Vrn-A1*, and had the largest effect size of all five loci ($w = 15.6$ for the unknown metabolite M501). Overall, most significant effects were found for *QZad-aww-7A* (26 metabolites). Interestingly, this is the only phenology-related locus for which effects on metabolites measured in negative ionization were stronger than for those measured in positive ionization mode. *QZad-aww-7D* affected 19 metabolic traits. Both *QZad-aww-7D* and *QZad-aww-7A* QTL affected many organic acids, whereas *Ppd-B1* and *Vrn-A1* affected many nucleosides, glucosides and their analogues.

Table 2
List of putatively annotated compounds measured by LC–QTOF-MS in positive ionizations (ESI$^+$) and negative ionization (ESI$^-$) mode (Supplemental Tables S1 and S2 for complete details).

Compound class	ESI$^+$	ESI$^-$	Total
Alkaloids	26	0	26
Amines	2	0	2
Amino acids, peptides, and derivatives	12	1	13
Carbohydrates	5	10	15
Fatty acids and conjugates	10	0	10
Flavonoids and derivatives	19	6	24
Glucosides	8	1	9
Nucleosides, nucleotides, and analogues	6	2	8
Organic acids	7	9	16
Organic compounds	9	1	10
Phenols	9	2	11
Phenylpropanoids and derivatives	7	5	12
(Hydro)quinones	3	0	3
Sugar acids and derivatives	3	0	3
Terpenoids	17	3	19
Lipids	7	0	7
Stilbenoids	2	0	2
Steroids, sterols and derivatives	5	0	3
Total number of putatively annotated compounds	157	40	194
Unknowns	312	49	361
Total number of all measured compounds	469	89	558

To gain a better understanding of the effects of the plant phenology-related loci on agronomic traits we plotted the Wald statistics of the five loci for the complete set of agronomic traits (Fig. 2, Supplemental Table S5; for a list of the description of all agronomic traits, see Table 3). Consistent with the results obtained by Edwards [19] for this experiment, no significant effects of *QZad-aww-7D* were detected. Wald statistics are much larger for agronomic traits than for metabolic traits for the other four loci. The numbers of significant effects on agronomic traits were 11 for *Vrn-A1*, 13 for *Ppd-B1*, 15 for *Eps*, and 24 for *QZad-aww-7A*.

Not surprisingly, four out of the five loci were found to have significant effects on two plant phenology traits, days to senescence and thermal time to heading (TTH). The strongest effects for these traits were observed for *QZad-aww-7A*, with Wald statistics of $w = 125.75$ for days to senescence and $w = 143.17$ for TTH. That locus also had strong effect sizes on harvest index ($w = 66.65$), grain yield ($w = 54.37$), grain size G2.5 ($w = 83.88$) and screenings L.2.2 ($w = 71.70$).

3.3. Genetic correlations among metabolic and agronomic traits

Many biochemically related metabolites measured in ESI$^+$ mode were more strongly genetically correlated than biochemically unrelated metabolites (Supplemental Table S6). For example, metabolites derived from adenosine (including adenosine monophosphate, 2′-deoxyadenosine, and 1-methyladenosine), amino acids and derivatives (including glutamate, pyroglutamic acid, 4-oxoproline, pipecolate, leucine, and isoleucine) and flavonoids and derivatives (lucenin-2, vitexin, violanthin, and rutin) were genetically correlated ($P < 0.05$), indicating common genetic factors controlling the levels of these metabolites.

As expected, inclusion of the genotypes of phenology-related loci in each of the linear mixed models reduced many of the correlations among agronomic traits and many of the correlations between agronomic and metabolic traits (Supplemental Tables S7, S8 and S9) compared with the results reported by Hill et al. [16].

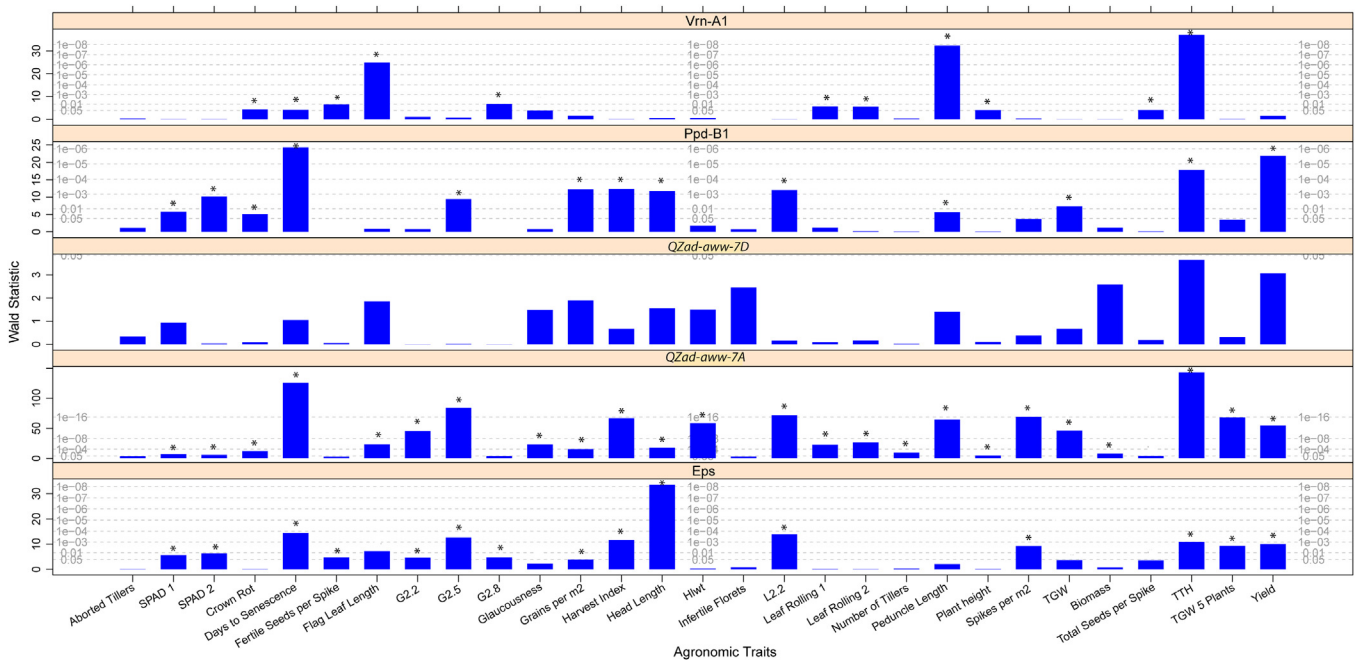

Fig. 2. Wald test statistic for the effect of each of the five phenology-related loci on the agronomic traits (see Supplemental Table S5 for complete data set). Horizontal lines indicate different significance thresholds of the Wald statistic. Significant effects ($P < 0.05$) are marked with an asterisk. G2.8, G2.5, G2.2: the percentage of grains greater than 2.8 mm, 2.5 mm, and 2.2 mm, respectively; Hlwt: Test weight; L2.2: screenings; SPAD_1: chlorophyll content at time point 1; SPAD_2: chlorophyll content at time point 2; TGW: 1000-grain weight; TGW_5P: 1000-grain weight of 5 plants; TTH: thermal time to heading.

The strongest positive correlation ($r_G = 0.42$) was between glaucousness and the unknown metabolite M625 (Supplemental Tables S8 and S9). Glaucousness was also positively correlated ($r_G = 0.36$) with the fatty acid M308 (putatively identified as tetradecenoic acid). The phenolic compound M6 (*n*-propyl gallate) exhibited moderate positive genetic correlation with both grains per square metre ($r_G = 0.32$) and grain yield ($r_G = 0.31$). The strongest negative correlation ($r_G = -0.34$) was found between the organic acid M567 (dehydroascorbate) and grain yield. The organic acid M565 (2-methyl-maleate) also exhibited a moderate negative correlation ($r_G = -0.33$) with grain yield. Both M567 and M565 were positively correlated with thermal time to heading.

Several metabolites exhibited negative genetic correlations with thermal time to heading and days to senescence. These included phenolic compounds such as M65 (epigallo catechin) and

Table 3
List of phenotypic traits measured for 179 lines of the Excalibur/Kukri doubled haploid population.

Class	Trait name	Abbreviation	Measure (units)
Phenological trait	Thermal time to heading	TTH	Thermal degree days (°Cd)
	Days to senescence	DS	Days
Physiological trait	Glaucousness	Gla	Visual scale from 1 (non-glaucous) to 9 (glaucous)
	Leaf rolling 1 and 2	Lfr1 and Lfr 2	Visual scale from 1 to 5 [72], measured at two time points
	Chlorophyll content 1 and 2	SPAD1 and SPAD 2	SPAD units, measured at two time points
Morphological trait	Flag leaf length	Flth	cm
	Head length	Hlth	cm
	Peduncle length	Plth	cm
	Plant height	Ht	cm
	Tillers	Tl	Number of tillers
	Aborted tillers	Atl	Number of aborted tillers
	Infertile florets	InfFl	Number of infertile florets
Agronomical trait	Crown rot symptom severity	CR	Scored visually from 1 (not infected) to 9 (90% infected)
	Thousand grain weight	TGW	Weight (g)
	Thousand grain weight (5 plants)	TGW5P	Weight (g)
	Fertile seeds per spike	Fsp	Number of fertile seeds per spike
	Grain yield	Yld	Yield (kg/ha)
	Seeds per spike	Sp	Number of seeds per spike
	Spikes per m^2	SpM2	Number of spikes per m^2
	Grains per m^2	Gm2	Number of grains per m^2
	Grain size 2.8	G2.8	Grain size larger than 2.8 mm (%)
	Grain size 2.5	G2.5	Grain size larger than 2.5 mm (%)
	Grain size 2.2	G2.2	Grain size larger than 2.2 mm (%)
	Screenings	L2.2	Material smaller than 2.2 mm (%)
	Test weight	Hlwt	Grain density (kg/hL)
	Harvest index	Hi	Weight of the grain as a percentage of the total weight (%)
	Total biomass	Bm	Weight (g)

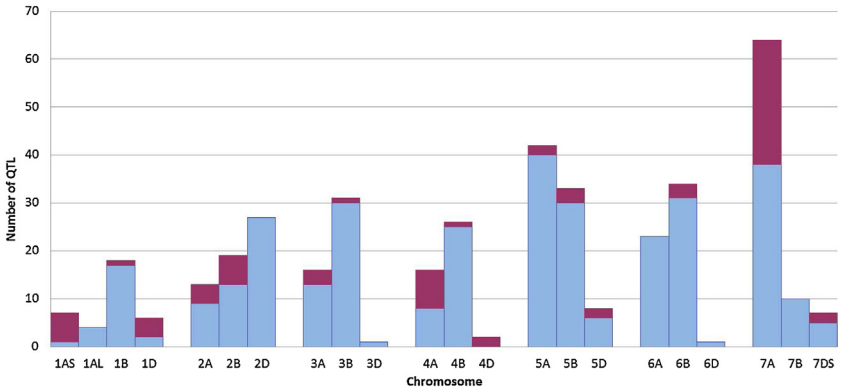

Fig. 3. Frequency distribution of the number of detected QTL ($P < 0.005$) for metabolic traits across the bread wheat chromosomes measured in both positive (blue) and negative (red) ESI modes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

M6 (*n*-propyl gallate,) and alkaloids such as M97 and M98 (both scoulerine) and M100 (capnoidine).

3.4. Chromosomal regions associated with QTL for metabolic traits

QTL were detected for 238 metabolites (Fig. 3, Supplemental Table S10). These mQTL were distributed across 159 intervals on the genetic map. The number of mQTL per metabolite ranged from one (for 125 metabolites) to nine (for M551 (malate)) with an average of two. Interestingly, QTL for organic compounds, nucleosides, phenols, flavonoids and derivatives, glucosides, terpenoids and alkaloids were almost exclusively found on either A or B genomes across all chromosomes, whereas a large majority of QTL for organic acids and carbohydrates were detected on the D genome.

To identify the proportion of metabolic variation that is genetically determined, we estimated broad-sense heritability (H^2) for each metabolite. For individual metabolites, the estimated broad-sense heritability ranged from 0.02 for an unknown compound (M498) to 0.77 for a flavonoid (M199, vitexin) (Supplemental Table S10) with a median of 0.54, indicating that a large proportion of the observed metabolic variability for many metabolites can be attributed to genetic variation, and can be highly heritable.

Different ionization modes show a different selectivity to ionize certain metabolites, depending on the affinity of a molecule to either accept or lose a proton using LC–MS analysis. Several metabolites were detected in both the ESI^+ and ESI^- modes. For most of these, similar mQTL were detected in both ionization modes. For example, the 3-cM interval between *barc0343* and *wPt-0538* on chromosome 4A was found to affect the concentration of the alkaloid tectoridin, as assessed in both the ESI^+ and ESI^- modes. Similarly, two adjacent intervals on chromosome 7A had the strongest ESI^+ and ESI^- effects for the organic acid, aconitic acid, and the flavonoid tricin.

3.5. Chromosomal regions associated with QTL for agronomic traits

QTL were detected for 10 agronomic traits (crown rot symptom severity, days to senescence, glaucousness, grains per square metre, harvest index, head length, test weight, peduncle length, plant height, and grain yield), with between one and eight QTL per trait (Supplemental Table S11). These QTL are distributed over 25 intervals on the genetic map. Some of them had quite large effects. For example, an interval on chromosome 7A was found to explain 49% of the genetic variation for grain yield, and one on chromosome 4A was found to explain 85.9% of the genetic variation for peduncle length.

3.6. Comparison of chromosome regions detected for both metabolic traits and agronomic traits within the Excalibur/Kukri doubled haploid population

Having mapped metabolic, agronomic, and phenology-related QTL to the Excalibur/Kukri genetic map, we investigated the level of co-regulation of these sets of traits by comparing the locations of their QTL. QTL for agronomic traits (crown rot symptom severity, days to senescence, grains per square metre, harvest index, head length, and grain yield) co-located with mQTL (Supplemental Tables S10 and S11).

The genome-wide distribution of agronomic and metabolic QTL in the doubled haploid population is illustrated in Fig. 4. Eight genomic regions affected both metabolic and agronomic traits (marked as regions I–VIII in Fig. 4): one each on chromosomes 2B (I), 4A (II), 6B (V) and 6D (VI), two on adjacent genetic markers on 5B (III and IV), and two on distant loci on 7A (VII and VIII).

On chromosome 5B, QTL affecting grains per square metre and levels of several metabolic features such as the phenolic compound *n*-propyl gallate and the amine *S*-acetyldihydro-lipoamide were detected in a 6.7-cM interval (*wPt-8604-wPt-5175[C]*). In an adjacent genomic region (*wPt-5175[C]-barc0216*), QTL were detected for grain yield and 15 metabolic traits, including the nucleoside 1-methyladenosine, the flavonoid 6,8-diprenylnaringenin, the phenylpropanoid archangelicin, and the alkaloid cuscohygrine. One locus on chromosome 7A (*wmc0283-1246868*) affected grain yield, harvest index, and days to senescence. This position coincided with QTL for several metabolites, such as the organic acids dehydro-ascorbate and 2-methyl maleate, and the flavonoid tricin. Notably, this QTL is adjacent to the 2.6-cM interval between *1246868* and *990491* harbouring QTL for 36 metabolic traits, including several organic acids (aconitic acid, 2-methyl maleate, dehydro-ascorbate), nucleosides (adenosine-5′-monophosphate, futalosine), and one phenylpropanoid (chlorogenic acid). A second genomic region on chromosome 7A, flanked by *ISBP_7A58* and *ISBP_7A49*, harbours a QTL for grain yield and the alkaloid scoulerine. Associations were also found between crown rot symptom severity and the organic acid 2-methyl maleate on chromosome 2B, as well as between grain yield and levels of phenylpropanoid chlorogenic acid on chromosome 4A.

Interestingly, the same effect sign (positive effects from Excalibur) was found for all metabolic QTL detected on chromosomes 3D, 6B and 7B. This suggests that on these chromosomes, Excalibur carries alleles of genes (transcriptional and/or other regulatory factors, or structural genes) that increase the levels of a number of metabolic traits. In contrast, for all mQTL found on chromosome 3B, the Kukri allele contributed a positive effect on the trait.

4. Discussion

4.1. Impact of phenology-related loci on metabolic and agronomic trait data

The major phenology traits in wheat, including the various components of maturity, are highly heritable and in some cases the genes underlying these loci have been cloned [29,30]. In contrast, traits such as grain yield are of low heritability and strongly influenced by the growth environment. Small changes in phenology can lead to large changes in the environmental impacts the plants are exposed to at critical developmental stages. This variation in environmental exposure not only results in major changes in many agronomic traits but also changes in metabolite levels.

In the first part of this study, we investigated the extent to which five phenology-related loci in bread wheat influence both metabolism and several agronomic traits to further develop our understanding of the importance of flowering time on plant traits, particularly its role in drought stress tolerance and avoidance. In this field experiment, Excalibur was earlier to reach 50% head

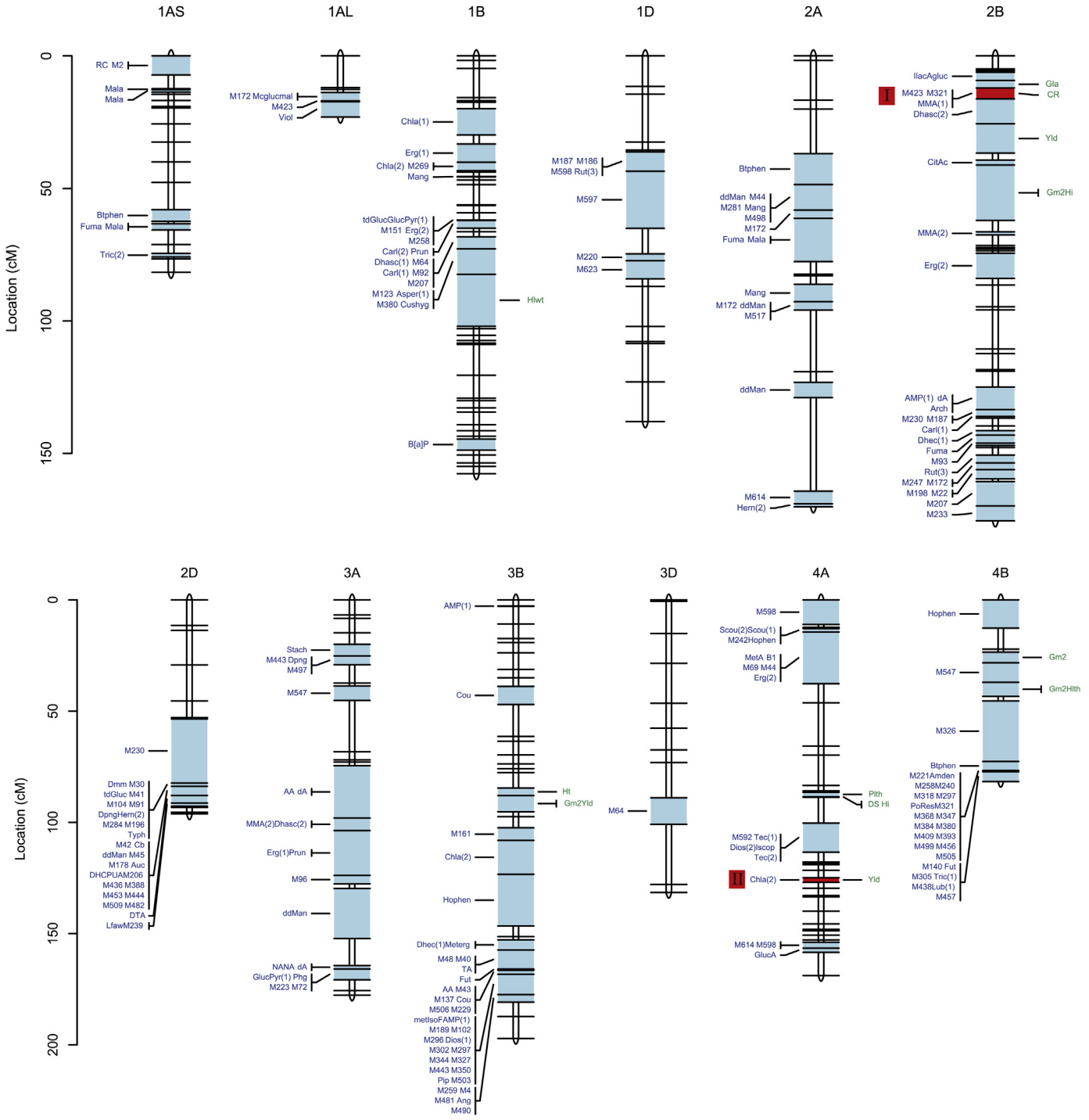

Fig. 4. QTL location on the cv Excalibur/Kukri genetic linkage map. Genetic distances are indicated as cM to the far left of the genetic map. QTLs for metabolites (with putative annotations were applicable) are shown on the left side, and QTLs for agronomic traits are shown on the right side, of each wheat chromosome (blue bars). For abbreviations of the metabolites and agronomic traits, see Supplemental Tables S10 and S11, respectively. Due to the larger number of mapped traits, chromosome 7A is shown on a larger scale to provide more detail. Genomic regions with several co-located QTL are indicated with red bars numbered from I to VIII.

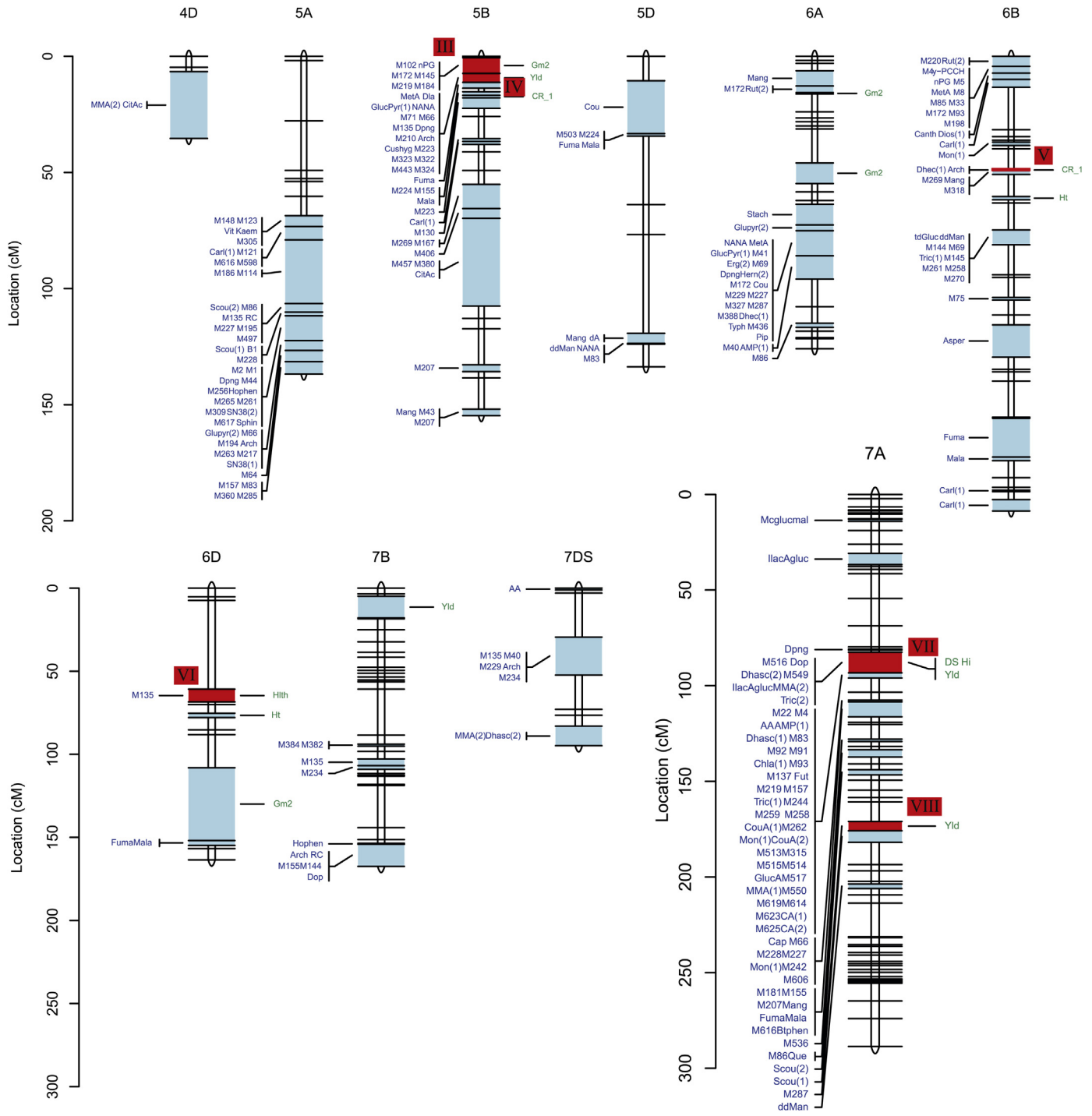

Fig. 4. (*Continued*).

emergence than Kukri [19], and 3 weeks variation in heading time was observed across the doubled haploid mapping population. To minimize the confounding effects of plant phenology, we adjusted the data prior to analysis of genetic correlations and QTL, by fitting five phenology-related loci as fixed effects in our model. The remaining genetic variation of the doubled haploid and parent lines is therefore a representation of the residual genetic component of the trait.

By estimating the size and significance of the phenology-related loci effects for each of the metabolic and agronomic traits the relative importance of *QZad-aww-7A* and 'earliness *per se*', in comparison *QZad-aww-7D*, vernalization requirement and photoperiod response (Figs. 1 and 2, Supplemental Tables 3–5), was determined. In wheat, the major genes affecting vernalization (*Vrn*) requirement and photoperiod insensitivity (*Ppd*) are located on chromosome 5A (*Vrn-A1*) [31] and 2B (*Ppd-B1*) [32,33]. *Ppd-B1* and *Vrn-A1* are sensitive to day length and cold temperature, respectively. Vernalization, the induction of flowering by prolonged exposure to low temperatures, is a major determinant of flowering time and *Vrn* genes affect physiological development and environmental adaptation in wheat plants, thereby influencing grain yield potential [34]. As the wheat mapping population was grown in a field environment classified as short day (10 hours or less) during grain filling [19], it was possible to observe significant differences in phenology

between photoperiod sensitive and insensitive genotypes. However, both *Ppd-B1* and *Vrn-A1* were found to have similar size effects of minor importance on primary metabolites (nucleosides, glucosides and their analogues) (Fig. 1, Supplemental Tables S3–S5), showing that metabolites detected in this study are not under major influence of these loci.

Eps loci influence flowering time irrespective of the environment. Edwards [19] has described the importance of an *Eps* QTL on chromosome 4A in this population. Here, that QTL was found to have stronger effects than *Ppd-B1* and *Vrn-A1* on metabolic traits and also had the largest effect of all maturity loci on one unknown metabolite. Based on these results it is not possible to distinguish whether this locus contains just one pleiotropic gene or several linked genes, each affecting one or more traits. Allelic variation at *Eps* loci such as this one could be used to fine tune flowering time irrespective of environmental conditions [35] and to manipulate the plant's metabolism to suit particular environments based on the genetic background of plant phenology-related loci. Studies such as ours show that genes underlying phenology-related loci have pleiotropic effects on other aspects of plant growth, development, and metabolism, which has important consequences for wheat breeding for specific adaptation to different environments.

Two additional loci affecting plant phenology (*QZad-aww-7A* and *QZad-aww-7D*) were considered in this study, both of which had been previously mapped by Edwards [19]. *QZad-aww-7A*, which affects responses to both photoperiod and vernalization treatments [19], is the only locus which showed significantly stronger effects on metabolites measured in negative ionization mode than those measured in positive ionization mode. This probably indicates that this locus differentially affects primary metabolites, such as many amino acids as well as organic acids, that were mostly detected in negative ionization mode, including glutamate, D-gluconic acid, 2-methyl maleate and threonate. For more complex secondary metabolites, which were mostly detected in positive ionization mode, *QZad-aww-7A* had relatively fewer significant effects and smaller effect sizes.

Differences in plant maturity have long been known to also influence agronomic traits. Reynolds and Tuberosa [36] and Pinto et al. [37] have discussed the confounding effects of phenology on QTL mapping studies of cereal crops. In this study, four of the five phenology-related loci were found to affect agronomic trait variation, with significant effects on more agronomic traits than metabolic traits. *Ppd-B1* had stronger effects than either *Vrn-A1* and or the *Eps* QTL on chromosome 4A, showing that day length was a major factor influencing agronomic traits in this experiment. These findings demonstrate that *QZad-aww-7A* had pleiotropic effects on both metabolic and agronomic traits.

Although not explored in this study, evidence is emerging that metabolites can drive some aspects of plant phenology, which should be considered and explored in future studies. Carreno-Quintero et al. [38,39] found association of primary metabolites, carbohydrates and amino acids, with the plant maturity region on the potato chromosome 5 and suggested that metabolites can influence the degree of plant maturity and *vice versa*. As another recent example, trehalose, an osmoprotectant, and its precursor trehalose-6-phosphate were shown to be involved as signalling metabolites in regulation of metabolism, plant growth and development in response to carbon availability [40]. Others [41,42] have shown that high sucrose levels play a critical role for controlling the transition to flowering, and Weber et al. [43] demonstrated a role for sucrose in differentiation and maturation. Pourcel et al. [44] found that flavonoids, part of the secondary metabolism, are involved as signalling metabolites and can alter the expression level of core pathway enzymes leading to the modulation of various developmental processes. As an example of how changes in secondary metabolism can affect circadian clock outputs, Kerwin et al. [45] showed that natural variation in a secondary metabolic enzyme associated with the regulation of aliphatic glucosinolates can alter circadian regulation of gene expression in Arabidopsis.

4.2. Associations of metabolites with agronomic traits

Recent studies have defined plant metabolites as potential biomarkers for variation in agronomically important phenotypes [46,47]. In this study, we used genetic correlations to link metabolic and agronomic traits. Genetic correlation among traits, which is due to some kind of shared genetic basis, either pleiotropic loci or linkage on the chromosome [46,48], can be used to predict how selection on one trait will lead to a correlated response in the other trait. Consistent with several published studies [38,49,50], many biochemically related metabolites measured in positive ionization mode were more strongly genetically correlated than were biochemically unrelated metabolites.

The genetic correlation coefficients between agronomic traits and between agronomic and metabolic traits were weaker than estimated in a previous study [16]. This is most likely due to inclusion of the five phenology-related loci in the statistical model, indicating that phenology effects are present in both metabolic and agronomic trait data. Despite showing either mostly weak or medium associations with agronomic traits, the roles of some known molecules and protectants involved in drought stress tolerance were highlighted in this investigation. For example, significant positive associations were found between the fatty acid punicic acid and glaucousness. Conjugated linolenic acids (here putatively annotated as punicic acid) are known to be present in plant leaf epicuticular waxes [51,52]. Glaucousness, which appears as an epicuticular wax composed of long chain fatty acids [53] on leaves, has been proposed as a trait to improve the grain yield of wheat in response to drought [54,55]. However, in this particular experiment, glaucousness was only weakly genetically correlated with grain yield.

Several phenolic compounds including phenolic acids showed higher levels in lines that headed early and produced high grain yield under drought. The phenolic compound *n*-propyl gallate is involved as a plastid terminal oxidase inhibitor in chloroplast respiration and has been shown to have an additive significant effect on the photosynthetic efficiency, and increased chlorophyll fluorescence levels in algae [56] grown under low salinity, and *Hibiscus rosa-sinensis* plants grown in water-limiting conditions [57]. *n*-Propyl gallate showed moderate significant genetic correlations with chlorophyll ($r_G = 0.18$), grains per square metre ($r_G = 0.32$) and grain yield ($r_G = 0.31$).

Most environmental stresses lead to disturbances in plant metabolism and cause oxidative injuries by enhancing the production of reactive oxygen species [58]. Phenolic compounds are known to confer protection with their antioxidant capacity against a range of physiological stresses including UV-B radiation, heavy metals, pathogen attack, and drought [59–62]. Increases of phenolics in wheat in response to drought have previously been reported [60,63]. Our results indicate that plant tolerance to drought may be associated with increased levels of these antioxidant constituents.

Previously, Hill et al. [18] found that *hkt1;1* mutant plants, which lack an important Na^+ transporter and are more affected by salinity than control lines, showed strong increases in the levels of TCA cycle intermediates in the shoots after salt treatment, coinciding with a significant depletion in sugar levels. Similarly, Kaplan et al. [64] found that levels of several TCA cycle intermediates, including fumarate and malate, as well as amino acids were increased after both heat and cold stress. In this study, and in the GC–MS results reported by Hill et al. [16], metabolites associated with the TCA cycle (aconitic acid, 2-methyl maleate, citrate and isocitrate) were associated with grain yield under drought.

Changes in levels of TCA cycle intermediates under abiotic stress may reflect a need for the plant to fundamentally change or redirect its carbon metabolism in response to environmental stresses that lead to cellular dehydration causing osmotic stress. To help the plant survive the stress, carbon influx into the TCA cycle (*e.g.* through glycolysis) is increased at a constant rate of C-efflux from the cycle (*e.g.* as CO_2 or usage of organic acids in other reactions, such as amino acid synthesis) to either generate more energy to regain homeostasis, or to serve as precursors for the synthesis of compounds that help the plant cope with water deficit (*e.g.* osmoprotectants).

4.3. Mapping of metabolic and physiological variation in wheat leaves

QTL studies in cereal crops have often been performed using parents that show strongly contrasting responses to certain stresses. However, as the parental lines often differ for major phenology genes, QTL associated with stress responses in such populations are likely to be confounded by differences in either flowering time or plant architecture, such as height. When the Excalibur/Kukri doubled haploid population was developed, markers for most vernalization and both major photoperiod genes were not available and the two parents showed similar maturity and carried the same dwarfing genes. However, they differed at phenology-related loci and this led to the phenological variation seen in the segregating population.

In the previous mQTL study, a large number of traits were associated with the marker loci on chromosomes 2B, 4A, 5A and 7A, which bear five plant phenology-related loci [16]. In this study, we included the positions of these five loci as co-variates in our analyses, preventing the detection of QTL at or near these loci. However, this cannot address the problem fully, since late developing lines can experience a very different environment compared to early maturing lines, thereby making comparisons difficult. Furthermore, QTL for days to senescence were detected even after including the five phenology-related loci in the model, which implies that those five loci do not fully explain the genetic control of maturity and that there may be other loci with minor effects that contribute to phenology. This study clearly illustrates the importance of selecting parents with the same alleles of genes for flowering time that are known not to be directly relevant to the traits of interest. Using progeny from an Elite × Elite cross expressing a restricted range of phenology has been shown to have low variation for flowering time and improved QTL detection [37,65].

Despite these challenges, the results of this and other recent studies indicate that using a combination of metabolomics and genetic/genomic research tools has a potentially high value for plant breeding because much of the variation in metabolites is heritable [66,67]. Yield traits with low heritabilities have been successfully incorporated into breeding programmes, showing promise for the incorporation of metabolic traits with average heritabilities of ~50% as found for plant secondary metabolites in this, and primary metabolites in the previous study [16]. In addition, QTL mapping of a diverse range of secondary plant metabolites of different metabolite classes, as reported here, could contribute to the identification of novel genomic factors underlying the genetic variation in secondary metabolite levels as part of a functional annotation of the wheat genome.

In conclusion, using genetic correlations and QTL co-locations, we identified sets of metabolites associated with particular traits belonging to distinct pathways, such as several phenolic acids, which were positively correlated with grain yield, and several organic acids found to be negatively correlated with grain yield. The QTLs detected for these metabolites were either at or near QTLs that affected grain yield and grain-yield related traits. As more of the wheat genome sequence becomes available, this information will be used to identify novel candidate genes involved in drought stress tolerance, and potentially provide tools to manipulate wheat metabolism or predict agronomically important traits based on metabolic composition.

Acknowledgements

The authors wish to thank the School of Botany (University of Melbourne) node of Metabolomics Australia (www.metabolomics.net.au) for access to the LC–MS facility, and Rudi Appels and Gabriel Keeble-Gagnère for useful comments and suggestions on the manuscript. This work was supported by the Australian Research Council and the Grains Research and Development Corporation; the South Australian Government; the University of Adelaide; the University of Queensland; the University of Melbourne; and by a Melbourne International Fee Remission Scholarship, a Melbourne International Research Scholarship, and a University of Melbourne Special Postgraduate Studentship to C.B.H.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2015.01.008.

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